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Plant colonization by GFP-labeled Bacillus amyloliquefaciens FZB42 and transcriptomic profiling of its response to plant root exudates

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Abbreviations

CLSM	confocal laser scanning microscopy
Cy3	Cyanine 3
Cy5	Cyanine 5
ECF	extracytoplasmic function
Em	erythromycin
FACS	fluorescence-activated cell sorting
FCH	fold change
FP	fluorescent protein
GFP	green fluorescent protein
IE	“interaction exudates”
ISR	induced systemic resistance
Km	kanamycin
LB	Luria-Broth
NRPS	nonribosomal peptide synthetase
OD	optical density
OD1.0	OD_{600}=1.0
OD3.0	OD_{600}=3.0
ORF	open reading frame
PCR	polymerase chain reaction
PGPR	plant growth-promoting rhizobacterium
PKS	polyketide synthase
RACE	rapid amplification of complementary DNA ends
RNAP	RNA polymerase
RE	root exudates
rpm	rounds per minute
SE	soil extract
SEM	scanning electron microscopy
sRNA	small RNA
TCS	two-component regulatory system
TEM	transmission electron microscopy
UTR	untranslated region
VOC	volatile organic compound
wt	wild type
1C	1C medium
1CS	1CS medium (1C medim+soil extract)

Abstract

In this work colonization of three different plants genera, maize, *Arabidopsis*, and *Lemna*, by GFP-labeled *Bacillus amyloliquefaciens* FZB42 in a gnotobiotic system was firtly studied using confocal laser scanning microscopy and electron microscopy. It was shown that FZB42 is able to colonize all these three plants with a specific pattern. Root hairs and the junctions where lateral roots occurred were a preferred area of FZB42 on both maize and *Arabidopsis* seedlings. On *Arabidopsis*, tips of primary roots were another favored site of FZB42; while, on maize, the concavities in root surfaces were preferred. FZB42 cells were also able to colonize *Lemna,* preferably accumulating along the grooves between epidermis cells on roots and the concaved intercellular space on fronds.

Secondly, microarray experiments were performed concerning the transcriptomic response of FZB42 to maize root exudates. A total of 302 genes representing 8.2% of FZB42 transcriptome were significantly altered in transcription by the presence of root exudates, the majority of them (260) were up-regulated in expression. The induced genes with known function were mainly involved in nutrition utilization, chemotaxis and motility, and antibiotic production.

The transcriptome of seven FZB42 mutants, defective in five sigma factor genes (*sigB, sigD, sigM, sigV,* and *sigX)* and two global transcriptional regulator genes (*degU and abrB*), were also investigated through microarray experiments. A vast number of genes were indentified to be controlled by the protein factors respectively. Possible mechanisms were proposed of how these protein factors are involved in the response to root exudates.

Finally, by northern blot existence of six out of 20 small RNA (sRNA) candidates was identified, which were significantly altered in expression by root exudates. This suggests that sRNA may play a hitherto unrecognized role in plant-microbe interaction.

Keywords:

PGPR, plant colonization, GFP, *Bacillus amyloliquefaciens* FZB42, microarray, root exudates

Zusamenfassung

In dieser Arbeit wurden zunächst die Kolonisationen von drei verschiedenen Pflanzengattungen durch den GFP-markierten *Bacillus amyloliquefaciens* FZB42 mittels confocaler Lasermikroskopie und Elektronmikroskopie verfolgt. Hier konnte gezeigt werden, dass FZB42 alle ausgewählten Pflanzen besiedeln konnte. Bei *Arabidopsis*- und Maiskeimlingen wurden die Wurzelhaare und Verbindungen, an denen laterale Wurzeln entstehen, durch FZB42 bevorzugt besiedelt. Weiterhin wurden bei *Arabidopsis* die Spitzen der Primärwurzeln, und bei Mais die Wurzelkerben bevorzugt besiedelt. Bei *Lemna* wurden FZB42 Zellansammlungen entlang der Furchen, die zwischen den Epidermiszellen der Wurzel liegen, sowie den intrazellulären Hohlräumen an der Blattunterfläche gefunden.

Anschließend wurden die Transkriptome von FZB42, der mit Maiswurzelexudat angezogen wurde, mittels Microarray analysiert. Insgesamt wurden 302 Gene, die 8,2 % des Transkriptoms ausmachen, signifikant durch das Wurzelexudat beeinflusst, wobei die Mehrzahl (260 Gene) hochreguliert wurde. Die induzierten Gene, dessen Funktion bereits bekannt ist, sind hauptsächlich an dem Nährstoffwechsel, Chemotaxis und Beweglichkeit, sowie an der Produktion von Antibiotika beteiligt.

Auch wurden die Trankriptome von sieben FZB42-Muatnten durch Microarray analysiert. Diese hatten jeweils eine Deletionen in fünf Sigmafaktor-Genen (*sigB, sigD, sigM, sigV,*and *sigX*) und zwei globalen Transkriptionsregulator-Genen (*degU* und *abrB*). Die Expression vieler Genen wird durch diese Genprodukte beeinflusst. Mögliche Mechanismen, wie diese Faktoren die bakterielle Reaktion auf Wurzelexsudaten beeinflüssen, wurden vorgeschlagen.

Schließlich wurden Northernblott-Untersuchungen an möglichen sRNA-Kandidaten durchgeführt, dessen Expression signifikant durch Wurzelexudate beeinflusst wurde. Dabei konnten 6 von 20 vermeintlichen sRNA-Kandidaten betätigt werden. Dies weist auf eine noch unbekannte Rolle der sRNAs bei der Pflanzen-Mikroben-Wechselwirkung.

Schlagworte:

PGPR, Kolonisierung an Pflanzen, GFP, *Bacillus amyloliquefaciens* FZB42, microarray, Wurzelexudaten

1 Introduction

1.1 Plant growth-promoting rhizobacteria (PGPR)

Plant growth-promoting rhizobacteria (PGPR) are generally defined as a heterogeneous group of bacteria which live in plant rhizosphere and contribute to plant growth [Lugtenberg *et al*. 2009]. In the last few decades a number of studies have been performed on the relationship between PGPR and their host plants [Vessey 2003; Preston 2004; Compant *et al*. 2005; van Loon 2007; Lugtenberg *et al*. 2009]. Several direct or indirect mechanisms have been elucidated as being involved in plant-beneficiary activities of PGPR, that include 1) synthesizing phyto-hormones such as indoleacetic acid, gibberellic acid, cytokinins and ethylene [Bloemberg *et al*. 2001; Idris *et al*. 2007; van Loon 2007] and volatile organic compounds [Ryu *et al*. 2003]; 2) producing available nutrients for plants [Vessey 2003; van Loon 2007], and 3) suppressing phytopathogenic soil bacteria, fungi, viruses and nematodes by production of antibiotics or other antimicrobial substances [Compant *et al*. 2005; Haas *et al*. 2005]. Some PGPR are also beneficial by eliciting plant response reactions directed against biotic ("induced systemic resistance", ISR) [van Loon 2007; Choudhary *et al*. 2009] or abiotic stress ("induced systemic tolerance", IST) [Yang *et al*. 2009]. At the same time, application of PGPR as biocontrol agent or biofertilizer has also intensively been investigated and some formulations are available as commercial products [Paulitz *et al*. 2001; Vessey 2003; Lucy *et al*. 2004; Compant *et al*. 2005].

To date, the preponderance of studies on PGPR have been conducted with Gram-negative bacteria, mostly on *Pseudomonas* spp., however, strains of *Bacillus* have also gained much attention due to an obvious advantages: *Bacilli* are able to produce heat- and desiccation-resistance endospores and, consequently, can be more easily stored and transported as stable products [Elizabeth *et al*. 1999; Bais *et al*. 2004; Kloepper *et al*. 2004; Francis *et al*. 2010].

Due to obvious differences in the physiology between G^+ and G^- bacteria, the two species may exhibit different mechanisms of plant-microbe interactions; however, compared with *Pseudomonas*, many aspects of G^+ PGPR still remain to be explored including both their lifestyles in rhizosphere and the molecular basis involved in their interaction with host plants.

1.2 *Bacillus amyloliquefaciens* FZB42

Bacillus amyloliquefaciens FZB42 is a Gram-positive PGPR, which has commercially been applied in a broad range of plants of economical importance. The whole genome sequence of FZB42 became available 2007 as the first representative of G^+ PGPR [Chen *et al*. 2007]. FZB42 has a relative "compact" genome of 3.918kb. It devotes as much as 8.5% of its whole genomic capacity to non-ribosomal production of antibiotics and siderophores. In the past several years consecutive studies have been performed with FZB42 in order to elucidate its plant growth-promoting and biocontrol activities [Idris *et al*. 2004; Koumoutsi *et al*. 2004; Butcher *et al*. 2006; Chen *et al*. 2007; Idris *et al*. 2007; Koumoutsi *et al*. 2007; Schneider *et al*. 2007; Chen *et al*. 2009; Ogata *et al*. 2009]. It was currently shown that the plant growth-promoting activity of this bacterium depends on at least the following several factors: 1) FZB42 is able to produce IAA, a plant growth hormone which stimulates cell elongation [Idris *et al*. 2004; Idris *et al*. 2007]. 2) Phosphate mobilization by the phytase secreted by FZB42 may provide a key nutrient under conditions of phosphate starvation [Idriss *et al*. 2002]. 3) Several antibiotics produced by FZB42 are found to be related with their biocontrol activity against plant pathogens [Koumoutsi *et al*. 2004; Chen *et al*. 2009].

1.3 Plant root colonization by PGPR

It is usually assumed that establishing an efficient colonization on plant roots is a critical step for PGPR for plant-microbe interactions [Chin-A-Woeng *et al*. 2000; Lugtenberg *et al*. 2001; Kamilova *et al*. 2005; Timmusk *et al*. 2005; Ongena *et al*. 2008]. A number of investigations demonstrated a non-uniform distribution of *Pseudomonas* on plant root: Some areas, including the extreme tip of the root, are practically free from bacteria whereas other areas can be highly colonized [Lugtenberg *et al*. 2001; Preston 2004] [Newman *et al*. 1974; Foster 1982; Fukui *et al*. 1994; Meharg *et al*. 1995]. In case of *Pseudomonas* the heavily colonized areas are usually found at junctions between epidermal root cells, concave parts of the epidermal surface, or sites where side roots appear [Bloemberg *et al*. 1997; Chin-A-Woeng *et al*. 1997], all presumed sites of exudation. Compared with *Pseudomonas*, however, so far only little was known about the colonization pattern of G^+ PGPR.

Except studies performed with classical approaches like light and electron microscopy, since more than one decade the green fluorescent protein (GFP) from jellyfish *Aequoria victoria* has been used as a valuable molecular marker for investigations of plant-microbe

interactions. As early as 1997, Bloemberg *et al.* reported about construction of plasmids which could stably maintain in *Pseudomonas* spp. and constitutively express a bright GFP fluorescence [Bloemberg *et al*. 1997]. Itaya *et al.* constructed a plasmid containing *gfp* for *Bacillus subtilis*, allowing detection of fluorescent *B. subtilis* colonies on agar plates [Itaya *et al*. 2001]. *Paenibacillus polymyxa* and *B. megaterium* tagged with plasmid-borne *gfp* have been used in studying plant root colonization [Timmusk *et al*. 2005; Liu *et al*. 2006]. Nevertheless, except for a few representatives of plasmids following theta replication, plasmids, especially their derivatives containing foreign DNA, are notoriously unstable in Bacilli [Ehrlich *et al*. 1986], limiting their use for constitutive expression of marker genes under environmental conditions.

1.4 Roles of plant root exudates in plant-microbe interaction

Plant roots secrete an enormous range of compounds, usually referred to as root exudates, into the rhizospheres. These root exudates are mainly carbon-containing compounds, which can often fall into two classes: low-molecular weight compounds such as organic acids, amino acids, sugars, phenolics and a variety of secondary metabolites, and high-molecular weight compounds like mucilage and proteins. It is estimated that pasture plants devote 30% and 50% of the total of photosynthates to roots and the allocation for cereals such as wheat and barley ranges between 20% and 30% [Yakov *et al*. 2000]. Typically, young seedlings exudate about 30–40% of their fixed carbon as root exudates [Whipps 1990].

There are increasing evidences that root exudates play a key role in plant-microbe interactions [Somers *et al*. 2004]. As early as at the beginning of 1900's, Hiltner had observed the abundant presence of microorganism in the rhizosphere, which was later found to be related with root exudation. It has been well-documented that bacterial communities in rhizosphere are less diverse [Marilley *et al*. 1998; Marilley *et al*. 1999] but of greater number [Whipps 1990; Semenov *et al*. 1999] than those present in distant bulk soil, an effect thought to be primarily resulted from the exudation by plant roots. With the advent of molecular biotechnology, more detailed relationships between rhizobacteria and root exudates were elucidated. For instance, Oger *et al*. [Oger *et al*. 1997] showed that genetically engineered plants (GEP) producing opines recruited 80% more opines-degrading bacteria of various species in their rhizospheres compared with non-GEP plants, probably because of an increased concentration of opines secreted by the GEP roots.

Rudrappa [Rudrappa *et al*. 2008] *et al*. demonstrated that root-secreted L-malic acid is involved in recruiting the beneficial rhizobacteria *B. subtilis* FB17 in a dose-dependent manner. More recently, Micallef *et al.* determined by T-RFLP and RISA that the root exudates patterns of eight *Arabidopsis* accessions exert a remarkable selective influence on bacteria associated with their roots [Micallef *et al*. 2009]. Generally, it is now widely accepted that root exudates provides not only abundant amount of carbon sources, which is usually a limiting factor for bacteria to propagate in soil, but also serve as signaling molecules which might trigger a series of microbial responses involved in plant-microbe communication [Badri *et al*. 2009]. It is noteworthy that, except for the mentioned benefit to rhizosphere microbes, plants roots also exude some anti-microbial compounds [Walker *et al*. 2003; Bais *et al*. 2005], which are basically thought to be the weapons of plants to expel or to prevent pathogenic microorganisms.

The release of root exudates are determined by plant species and also affected by the age and the physiological status of an individual plant as well as the factors like biotic and abiotic environments [Wieland *et al*. 2001; Buyer *et al*. 2002; Kowalchuk *et al*. 2002; Kuzyakov *et al*. 2003; Broeckling *et al*. 2008]. It has been demonstrated that some non-pathogenic strains of *Pseudomonas syringae* induce more low-molecular mass compounds while block synthesis or release of antimicrobial compounds from *Arabidopsis* roots [Bais *et al*. 2005]. In conclusion, quality and quantity of plant root exudates affect the microbial community in the rhizosphere; *vice versa*, the rhizobacteria can also influence the production of root exudates.

1.5 Using DNA microarray to study gene expression

Since the first description of using cDNA microarray to analyze gene expression in 1995 [Schena *et al*. 1995], this technology has rapidly been used in research community. In recent years, this method becomes more widely available for gene expression investigations. Compared with other methods such as suppression subtractive hybridization (SSH) and mRNA differential display, the advantage of microarray technology mainly lies in its capability to process quickly a huge amount of data obtained from different comparisons by using computer-aid analysis tools. This high throughput ability is particularly useful in handling the data from the whole transcriptome of a given organism.

The principle of DNA microarray is that a mixture of labeled DNA molecules hybridize specifically to the probes with a complementary sequence immobilized on a solid

surface, thus facilitating quantitative measurement of a vast array of sequences simultaneously [Brown *et al*. 1999; Southern *et al*. 1999]. The solid substrates providing a surface to be spotted with DNA probes usually include glass, e.g. Affymetrix chips, nylon membranes, gold coated slides and other materials [van Hal *et al*. 2000]. Besides using cDNA clone as probes on an array, oligonuclotides of a length of 20~70bp can also be synthesized directly, or after synthesizing, "printed" on a microarray chip.

A typical microarray experiment involves procedures as followed. Firstly RNAs are prepared from the two samples to be compared and then converted into cDNA by reverse transcription. Secondly, the two sets of cDNA mixture are labeled with a green fluorescent dye Cyanine 3 (Cy3) and a red fluorescent dye Cyanine 5 (Cy5) respectively. The labeled cDNAs are subsequently mixed and hybridized to a single microarray slide. Finally the slide is scanned and each spot onside is measured for the signal intensities of both dyes. The recorded images and data are store in a database and can later be analyzed with corresponding softwares.

Routinely, the logarithm of the ratio of Cy5 intensity to Cy3 intensity is calculated for each spot. A positive value of log (Cy5/Cy3) ratios indicates more Cy5-labeled transcripts in the sample mixture than the Cy3-labeled ones, whereas a negative value log (Cy5/Cy3) ratios indicates relative excess of the Cy3-labeled transcript in the sample. A value near to zero suggests an approximately equal abundance in the two samples. These logarithm values can easily be converted into the fold change, often used in many researches, of a transcript in one sample compared with that in the other sample. A fold change value indicates more intuitively the alteration magnitude of the transcription level of a gene between the two samples being compared.

Besides the two-color (or two-channel) microarray as described above, one-color (single-channel) system produced by several microarray manufacturers are also popular in practice, such as the Affymetrix "Gene Chip", Agilent single-channel arrays, and the Applied Microarrays "CodeLink" arrays. In one-color microarray system, the two cDNA samples are labeled with the same dye, usually Cy3 [Fare *et al*. 2003], and hybridized to two separate arrays. Then the signal intensities measured, respectively, from the two arrays were compared. The fact that each array chip is exposed to only one sample allows an aberrant sample not to affect the raw data derived from other samples. Another benefit is that the data obtained in this way are more easily to be compared across arrays. However,

the disadvantage of the one-color system is that it needs twice as many microarrays as the two-color system to compare samples within an experiment.

Along with microarrays becoming more broadly accessible to the researcher, various statistical analysis methods have quickly been developed, tackling with a series of drawbacks or features inherent to this system [Kerr *et al*. 2000; Tseng *et al*. 2001; Rosenzweig *et al*. 2004; Boorsma *et al*. 2005; Tang *et al*. 2007; Roberts 2008]. Since no "best method" can be determined, finding a most suitable method to the system used is often a wise choice in practice. Usually, a common theme in these approaches is to identify the significantly differentially expressed genes between two sets of samples, which is always of great importance to biologists. Apart from this goal, now searching for co-expressed genes by the method like hierarchical clustering is normally also included in most of microarray analysis softwares [Eisen *et al*. 1998; Boorsma *et al*. 2005].

An important application of DNA microarray is to study host-microbe interaction for the gene expression response of one side to the other [Diehn *et al*. 2001; Wan *et al*. 2002; Han *et al*. 2004; Wang *et al*. 2005; Graham *et al*. 2006]. For example, by DNA microarrays thousands of microbial gene expression can be monitored simultaneously during infecting the host, which helps us to examine physiologic adaptations of the microbes to various environmental conditions during infection, to predict the functions of uncharacterized genes and to identify novel virulence-associated genes. Except for pathogenic microbes, the interaction between beneficial bacteria such as PGPR and their hosts has been also investigated by microarray. G. Louise Mark *et al.* indentified several previously uncharacterized genes of *P. aeruginosa*, involved in competitive ability in the rhizosphere, by transcriptome profiling of *P. aeruginosa* in response to sugar-beet root exudates [Mark *et al*. 2005]. Another study using root exudates suggested that availability of particular nutrients, especially amino and aromatic compounds, is an important driving forces for *P. putida* to colonize the rhizosphere [Matilla *et al*. 2007].

1.6 Sigma factors of *Bacillus*

In prokaryotes transcription and translation occur simultaneously due to lacking of a nuclear membrane. Consequently, in contrast to the multilevel control occurring nearly equivalently in eukaryotes, the regulation of gene expression in bacteria happens primarily at the level of transcription. The holoenzyme of bacterial RNA polymerase (RNAP) is comprised of two parts, a catalyzing core enzyme consisting of two alpha (α), one beta (β),

one beta-prime (β'), and one omega (ω) subunit(s) and an additional sigma factor, which allow the holoenzyme to recognize promoter elements and initiate transcription from these sites. Upon the initiation of transcription, the sigma subunit binds with the core PNAP and determines most, if not all, of the specificity of the RNAP holoenzyme for its cognate promoter. It is generally believed [Carter *et al*. 1986; Carter *et al*. 1988; Haldenwang 1995] that this association is transient and after initiating the sigma factor is discharged from the core RNAP, although a recent study [Kapanidis *et al*. 2005] has shown that σ^{70} in E. coli remains attached in complex with the core RNAP, at least during early elongation. Throughout this introduction, an individual RNAP holoenzyme is referred as E-σ^{X}: E represents core RNAP, and σ^{X} represents the particular factor that it carries.

Much of our knowledge about the interactions between RNAP and promoters was obtained from experiments with *Escherichia coli*. It is assumed that the knowledge is directly applicable to the *B. subtilis* enzyme, although the RNAPs from these bacteria are not identical. As a most well-investigated representative of G^{+} bacteria, *B. subtilis* posses at least 17 distinct sigma factors [Yoshimura *et al*. 2004], seven of which (SigM, SigV, SigW, SigX, SigY, and SigZ) are members of the extracytoplasmic (ECF) subfamily. In comparison, the genome of *B. amyloliquefaciens* FZB42 encodes 16 sigma factors, six of which (SigM, SigV, SigW, SigX, YlaC and RBAM00641) are predicted to have extracytoplasmic function [Chen *et al*. 2007]. Whilst five of its six ECF sigma factor have a counterpart in *B. subtilis* 168, FZB42 possess a novel putative σ factor, RBAM00641.

The functions of sigma factors have not been fully explored; however, researchers have so far elucidated some of their functions, which are shortly discussed below.

1.6.1 Sigma factor σ^{A}

Sigma A is the first sigma factor isolated from purified RNAP in vegetatively growing *B. subtilis* [Shorenstein *et al*. 1973], probably because it is also the most abundant sigma factor and amenable to the techniques applied for its *E. coli* counterpart σ^{70}. The σ^{A} protein shows a molecular mass of 55,000Da in SDS-polyacrylamide electrophoresis [Shorenstein *et al*. 1973; Shorenstein *et al*. 1973]. The structural gene for σ^{A} is organized in an operon consisting of three genes: P^{23}, *dnaG* and *rpoD (sigA)*. While *dnaG* and *rpoD (sigA)* are essential for growth, the function of P^{23} remains elusive. The *sigA* operon is directed by six promoters, two of which are transcribed by SigA itself.

Typically, σ^A drives the main part transcription events in exponentially growing cells and is therefore a housekeeping sigma factor. In addition, σ^A is also involved in the expression of some specific genes which, for example, are required for heat shock response, synthesis of degradative enzymes of stationary-phase function, the development of competence for DNA transformation as well as early sporulation [Cheo *et al*. 1991; Li *et al*. 1992; Wetzstein *et al*. 1992; Chang *et al*. 1994; Haldenwang 1995]. Furthermore, there are also some controversial reports that σ^A plays an undefined role in late sporulation [Segall *et al*. 1974; Tjian *et al*. 1974].

1.6.2 Sigma factor σ^B

σ^B was the first alternative sigma factor detected in *Bacillus* and originally identified as a subunit of an RNAP holoenzyme transcribing a cloned sporulation gene (*spoVG*) *in vitro* [Haldenwang *et al*. 1979]. Like σ^A, σ^B was also demonstrated to be primarily present in vegetatively growing and early sporulating cells, although its amount is not more than 5% of the level of σ^A [Haldenwang *et al*. 1979; Haldenwang *et al*. 1981]. The structural gene encoding σ^B is the third one in a four-gene-operon (*rsbV-rsbV-sigB-rsbV*) which is directed mainly by σ^B itself [Kalman *et al*. 1990].

More than 70 genes were reported to be transcribed by E-σ^B [Sierro *et al*. 2008]. The transcription of σ^B-regulated genes is induced by several different environmental stress conditions such as heat shock, ethanol shock, oxygen limitation, high salt. Nevertheless, it is described that the genes which are transcribed by E-σ^B in response to environmental stresses have also additional promoters that are recognized by other RNAP holoenzymes [Haldenwang 1995]. Furthermore, some genes controlled by σ^B have been tested showing that they play a non-essential role in the growth of *B. subtilis*. Therefore it is argued that σ^B is a general stress sigma factor, probably participating or enhancing the stress responses but not essential to them [Haldenwang 1995].

1.6.3 Sigma factor σ^D

σ^D was identified as a novel sigma factor in 1988 showing 28,000Da in SDS polyacrylamide gel [Helmann *et al*. 1988]. The accumulation of σ^D peaks at late exponential phase, where 220 ± 50 molecules per cell are present in *B. subtilis* [Helmann 1991]. This abundance is approximately comparable to that of σ^B. The SigD gene of *B.*

subtilis locates near the 3' end of *fla-che* operon consisting of more than 30 genes responsible for flagellar or chemotaxis function. σ^D is primarily involved in transcribing the genes for flagellin synthesis (*hag*) [Mirel *et al*. 1989], methyl-accepting chemotaxis [Marquez *et al*. 1990], and autolysin synthesis [Marquez *et al*. 1990; Kuroda *et al*. 1993]. The unique consensus of σ^D –recognized sequences allows to search for genes with an upstream σ^D promoter [Helmann 1991], thereby leading to the identification of σ^D-like promoter upstream of *degR* and *epr,* respectively [Helmann 1991]. More genes, which were identified as SigD-regulated genes in *B. subtilis* by mean of DNA microarray and northern blotting, were also found to possess a promoter with a σ^D –recognized sequence [Serizawa *et al*. 2004].

1.6.4 Extracytoplasmic function (ECF) sigma factors

Extracytoplasmic function (ECF) sigma factors was originally proposed in 1994 by Lonetto *et al*. due to a common feature of their involvement in cell envelope functions (transport, secretion, extracytoplasmic stress) [Lonetto *et al*. 1994]. Besides this point, more shared features were later elucidated to refer them as an important subfamily distinct from other σ factors [Lonetto *et al*. 1994; Helmann 2002]. For example, their recognized promoters often share a highly conserved AAC motif at the -35 region and a GGT motif at the -10 region. And they usually function in a mechanism associated with a co-transcribed anti-σ factor, which possesses a transmembrane sensory C-terminal domain and an intracellular inhibitory N-terminal domain. These may also explain the regulatory overlap and functional redundancy among the ECF σ factors which are observed in many cases [Mascher *et al*. 2007]. *B. subtilis* has seven ECF σ paralogues, four of which (σ^X, σ^W, σ^M and σ^Y) have been investigated in detail while the roles of the other three (σ^V, σ^Z and σ^{Ylac}) remain unclear.

1.6.4.1 Sigma factor σ^X

σ^X is the first ECF σ factor subjected to an detailed investigation [Lonetto *et al*. 1994; Huang *et al*. 1997; Huang *et al*. 1998]. A *sigX* mutant strain displays an impaired ability to survive at high temperature [Huang *et al*. 1997] while an enhanced sensitivity to cationic antimicrobial peptides [Cao *et al*. 2004]. The σ^X regulon is strongly induced by cell wall antibiotic which inhibit peptidoglycan biosynthesis and tunicamycin, a specific inhibitor of wall teichoic acid synthesis [Helmann 2002]. Several genes as well as operons such as *dlt*

operon and *pssA* operon, that affect the composition or metabolism of cell envelope, are preceded by a σ^X –dependant promoter. A model of σ^X regulating cell envelope through affecting the overall net charges has been postulated [Helmann 2002]. More recently, σ^X is also demonstrated to be involved in controlling *B. subtilis* biofilm architecture through the AbrB homologue Abh [Murray *et al*. 2009].

1.6.4.2 Sigma factor σ^W

σ^W is typically induced by various cell wall stresses such as exposure to antibiotics, alkaline shock [Cao *et al*. 2001; Wiegert *et al*. 2001; Cao *et al*. 2002; Pietiainen *et al*. 2005]. To date, more than 30 different operons in *B. subtilis* have experimentally been established to be regulated by σ^W, some of which were known to mediate an intrinsic resistance to antimicrobial compounds produced by other *Bacilli* [Butcher *et al*. 2006].

1.6.4.3 Sigma factor σ^M

Expression of σ^M is up-regulated in response to a series of environmental stresses including high osmosis, heat shock, ethanol, acid, paraquat, phosphate starvation, cell wall antibiotics such as bacitracin, vancomycin, and cationic antimicrobial peptides, while it was not induced by alkali (pH 9), 5mM H_2O_2, the detergents such as 0.1% Triton X-100 and 0.1% Tween 20, or 50 μM monensin [Thackray *et al*. 2003] [Cao *et al*. 2002] [Horsburgh *et al*. 1999] [Thackray *et al*. 2003; Pietiainen *et al*. 2005]. It has also been reported that σ^M as well as σ^X are required for septum and teichoic acid synthesis in *B. subtilis* strain W23 [Minnig *et al*. 2003].

The ECF sigma factors, σ^M, σ^W, and σ^X, respond to a partially overlapping but distinct spectrum of stresses. For example, σ^X and σ^W show no obvious response to pH homeostasis or heat shock, while σ^M does [Hecker *et al*. 2001; Thackray *et al*. 2003]; σ^W and members of its regulon are induced by alkali stress [Wiegert *et al*. 2001] but σ^M not. Moreover, the three ECF sigma factors are active in different growth phase: whilst σ^X and σ^W are expressed in early stationary phase, σ^M is most active in early to mid-logarithm growth phase although it may also play a role in transient phase [Thackray *et al*. 2003]. Therefore Thackray *et al*. argued that both σ^X and σ^W, although recognizing different extracytoplasmic signals, are involved in mediating adaptation to compounds toxic to cell wall or membrane; in contrast, σ^M is required for cell maintenance under conditions of salt, acid, and ethanol stress[Thackray *et al*. 2003].

1.6.4.4 Sigma factor σ^Y

Compared with the other three ECF σ factors described above, the physiological role of σ^Y remains elusive. Several target operons were proposed to be regulated by σ^Y, however, only one of them, *ybgB* encoding for a hypothetical immunity protein against toxic peptides, was unambiguously identified as a direct target for σ^Y [Cao *et al*. 2003]. So far, no regulatory overlap was observed between σ^Y with other ECF σ factors [Mascher *et al*. 2007].

1.7 AbrB and DegU, two important global transcriptional regulators

Apart from sigma factors, other DNA-binding proteins (transcriptional repressors and activators) also modulate the efficiency of transcription in bacteria under specific stress conditions or during growth transitions and morphological changes. For instance, in *E. coli* a pool of more than 300 transcriptional regulators can be chosen to fine-tune the transcriptions within a cell [Perez-Rueda *et al*. 2000], while in *B. subtilis* a collection of 237 DNA-binding transcription factors was identified by a genomic approach, half of which have been experimentally evidenced [Moreno-Campuzano *et al*. 2006]. Among the regulators in *B. subtilis*, DegU and AbrB represent two most important general transcriptional factors, which have extensively been investigated.

1.7.1 AbrB

Upon entry into stationary phase from exponential-growth phase, bacteria have to coordinate a large number of genes to adapt to the environmental changes. This adaption is orchestrated under several so-called transition state regulators (TSRs), among which AbrB is one of the most widely studied. The transcription of more than 60 genes have been reported to be regulated by AbrB [Xu *et al*. 1996], most of which such as *comK*, *spoVG*, *phyC*, *aprE* and *abrB* itself are negatively regulated by AbrB, while only a few of which such as *citB* and *hpr* are positively regulated [Makarewicz *et al*. 2008; Sierro *et al*. 2008].

Although AbrB is a crucial transition state regulator, the transcription of *abrB* starts during vegetative growth. Moving into transition and subsequent sporulation growth, the transcription of *abrB* is repressed by the Spo0A protein [Strauch *et al*. 1990; Hahn *et al*. 1995; Greene *et al*. 1996]. So far no obvious conserved sequence has been found to be

specifically recognized by AbrB. Instead, the studies suggested that AbrB may, being a tetrameric form, recognize a general DNA tertiary structure [Vaughn *et al*. 2000; Bobay *et al*. 2004]. As a consequence, despite of a wealth of accumulation of biochemical and genetic data on AbrB, the general and specific mechanisms of how this DNA-binding protein plays its biological role remain elusive.

1.7.2 DegU

DegU is another global transcriptional regulator in *B. subtilis*, primarily controlling protein expression during post-exponential growth. DegU together with DegS comprise a typical member of the two-component system family employed by *B. subtilis* to respond environmental stimuli. In this system DegS anchors on membrane as a sensory histidine protein kinase while its cognate part DegU locates cytoplasmically. DegS exhibited both kinase and phosphatase activities [Tanaka *et al*. 1991], therefore allowing the autophosphorylation of its own histidine residue. Upon receiving a signal from the extracellular environment, the phosphoryl group is then transferred to the aspartate residue of the cognate response regulator DegU. As a transcriptional regulator, DegU coordinates expression of a number of genes in response to environmental changes. The genes regulated by DegU include those involved in genetic competence, synthesis of degradative enzymes and multicelluar behavior such as swarming motility, biofilm formation, complex colony architecture[Dahl *et al*. 1992; Dubnau *et al*. 1994; Kunst *et al*. 1994; Stanley *et al*. 2005; Verhamme *et al*. 2007; Murray *et al*. 2009]. According to recent genome-wide transcription and proteomic studies, more than 170 genes, accounting for ~4% of the *B. subtilis* genome were identified to be regulated by DegU under various growth conditions [Ogura *et al*. 2001; Mader *et al*. 2002; Murray *et al*. 2009].

DegU can serve its regulatory activity in two states: unphosphorylated and phosphorylated, although the latter is the main functional form in most cases. Phosphorylated DegU (DegU~P) is found to activate the expression of more than 120 genes [Tsukahara *et al*. 2008]. The functional mechanisms identified in this case include DegU~P recruiting RNA polymerase at the specific promoter regions of the genes like *yvcA* and *aprE* [Ogura *et al*. 2003; Verhamme *et al*. 2007]. However, the well-defined DNA recognizing sequence of DegU~P has not yet been identified. Not many target genes of unphosphorylated DegU have been identified; nevertheless, it is well known to be required for genetic competence by affecting the expression or activity of ComK, a master

regulator of competence development. Unphosphorylated DegU not only regulates the transcription of ComK by binding to its promoter region but also facilitates the autoregulation of ComK [Grossman 1995; Hamoen *et al*. 2000]

Because DegU functions depending on its phosphorylation by DegS, it used to be regarded as 'a molecular switch' that controls cell fate [Dahl *et al*. 1992]. However, recent studies suggest that DegU~P serves more like a 'rheostat' that, in response to environmental changes, triggers a series of processes along an increasing gradient of DegU phosphorylation [Kobayashi 2007; Verhamme *et al*. 2007]. Murray *et al*. summarized that DegS–DegU system is finely tuned at a three-tiered control within cells: *degU* transcription, DegU phosphorylation and DegU~P activity [Murray *et al*. 2009].

1.8 Small regulatory non-coding RNA in bacteria

Small non-coding RNAs in bacteria are usually referred to as 'small RNAs' (sRNAs), representing a heterogeneous group of RNAs of around 50~500bp in length, which are generally not translated but, in many cases, function as a regulator [Gottesman 2005; Altuvia 2007; Vogel *et al*. 2007; Waters *et al*. 2009]. Although the existence of small RNAs in bacteria has been discovered since 1970s, they have not gained a significant appreciation until recent years. As bacterial genome sequences are increasingly published, more and more small RNAs were detected firstly by a systematic research and then confirmed experimentally [Axmann *et al*. 2005; Landt *et al*. 2008; Swiercz *et al*. 2008; Arnvig *et al*. 2009; Perez *et al*. 2009; Preis *et al*. 2009; Saito *et al*. 2009]. Regarding the model microorganisms, for example, approximate 80 small RNA transcripts in *E. coli* and more than a dozen in *B. subtilis* have been verified [Kawano *et al*. 2005; Saito *et al*. 2009; Waters *et al*. 2009]. At the same time, the regulatory functions and mechanisms of several small RNAs in *E. coli* or *Salmonella* are also unveiled, shedding a light upon this vast but still mysteries world [Bouvier *et al*. 2008; Gorke *et al*. 2008; Repoila *et al*. 2009; Waters *et al*. 2009].

The small regulatory RNAs can be divided into three groups in light of their functional mechanisms. The simplest ones are riboswitches, which locate at the 5'-UTR end of mRNAs and respond to a target small molecule ligands [Mandal *et al*. 2004; Grundy *et al*. 2006; Montange *et al*. 2008]. In the presence of these metabolite signals, the riboswitches could adopt different conformation, thus terminating (by forming a terminator structure) or allowing (by anti-termination) the process of transcription, or regulating the

translation of a gene by switching the accessibility of a ribosome-binding site (RBS) [Waters *et al*. 2009]. Other *cis*-acting sRNA regulatory elements, for examples, RNA thermometer, can also be included in this group. RNA thermometer is a regulatory strategy used in bacteria in response to temperature fluctuations. Most of the known RNA thermometers are located in the 5'-UTR and mask RBS by forming a complex structure via base-paring at low temperatures. As temperature increases, the structure melts permitting ribosome access and translation initiation [Narberhaus *et al*. 2006; Digel *et al*. 2008].

A second group includes several proteins-binding sRNAs (RNase P, tmRNA, 4.5S, 6S, CsrB and GlmY), which act via modulating protein activity. For example, *E. coli* 6S sRNA affects gene expression in a fashion to antagonize the activity of RNA polymerase. In stationary phase, 6S sRNA is highly abundant in a cell and able to bind the housekeeping holoenzyme form of RNA polymerase, i.e., σ^{70}-RNA polymerase, therefore inhibiting the initiation of many genes' transcription. Whereas 6S does not form stable complexes with σ^{S}-RNA polymerase, an important form of RNA polymerase during stationary phase, as was shown by both *in vitro* and *in vivo* experiments [Trotochaud *et al*. 2005; Wassarman 2007]. Therefore, 6S sRNA is able to regulate the transcription of some genes, at least partially by affecting the competition between the two forms of RNA polymerase for the specific promoters recognized by σ^{70} –RNAP or σ^{S}-RNAP.

The third group comprising the majority of characterized sRNAs regulates gene expression by base pairing with mRNA. These sRNAs are antisense, with an extensive or a limited complementary sequence, to their target genes. They can be *cis*-encoded on the opposite strand of their target genes or *trans*-encoded, many residing in inter-genetic regions, distant from their target genes. Base-paring of a sRNA with its target mRNA at Shine-Dalgarno sequence, AUG start codon or 5' mRNA coding region can inhibit the occurrence of translation and often leads to the degradation or cleavage of the target mRNA [Gorke *et al*. 2008]. On the contrary, some sRNAs can act positively by preventing their target mRNA from the formation of an inhibitory structure, which sequesters the RBS [Waters *et al*. 2009]. It is intriguing that the regulation of all trans-encoded sRNA characterized so far required Hfq, a RNA chaperon, which is shown to facilitate the RNA-RNA base-paring and/or modulating sRNA level.

Although the functions of most sRNAs are not yet understood, the known evidences revealed that, in general term, the sRNAs mediate the response to various environmental cues or stresses [Waters *et al*. 2009]. As mentioned above, riboswitches control

biosynthetic genes by sensing different concentrations of their target metabolites, while the 6S and CsrB families of sRNAs regulate the expression of a large number of genes in response to altered nutrient availability. The *trans*-encoded sRNAs are mainly known to enhance bacterial ability to adapt to environmental stimuli. For example, sRNA SR1 repress the translation initiation of AhrC, a negative transcription regulator of arginine metabolism expression [Heidrich *et al*. 2006; Heidrich *et al*. 2007]. Another sRNA Spot42 specifically binds to the 5' region of *galK* mRNA and blocks the binding of 30s ribosome so that the translation of GlK is inhibited under unnecessary physiological conditions [Moller *et al*. 2002]. Particularly, a set of *trans*-encoded sRNAs are involved in modulating of the nature and abundance of envelope components to survive in a changing environment. These sRNAs regulate outer membrane proteins (MicA, MicC, MicF, RybB, CyaR, OmrA, and OmrB) or transporters (SgrS, RydC, and GcvB), which in return are able to control the utilization of some sugar and other intermediates. In addition, the functions of the small RNAs are also associated with iron homoeostasis, quorum sensing, as well as the virulence of some pathogenic microorganisms [Romby *et al*. 2006; Toledo-Arana *et al*. 2007; Repoila *et al*. 2009; Waters *et al*. 2009].

Actually, regulation through sRNAs is often considered to be more cost-effective than through regulatory proteins, because these molecules are small and do not need to be translated, and therefore the energetic cost of their synthesis is smaller in comparison to regulatory proteins [Altuvia *et al*. 2000; Guillier *et al*. 2006]. This view has gained support through quantitative modeling the regulation of gene expression by sRNAs [Shimoni *et al*. 2007]. Moreover, gene regulation through sRNA exhibits features that can be not achieved by proteins [Levine *et al*. 2007].

1.9 Research objectives

Although two investigations have been reported of transcriptomic response of Gram-negative *Pseudomonas* spp. to root exudates [Mark *et al*. 2005; Matilla *et al*. 2007], none of such research has been performed with Gram-positive PGPR. Since a number of differences exist between G^+ and G^- bacteria in the known physiology, and probably also in the mechanisms of plant-microbe interactions, an elaborate study is of great interest on the transcriptional response of G^+ PGPR to the signals from plant roots. To accomplish such a goal, *Bacillus amyloliquefaciens* FZB42 was used taking advantage of the availability of its complete genome annotation data and the steadily progressing knowledge concerning

its interaction with plants. The project was performed in collaboration with the CeBiTec in Bielefeld, who was in charge of microarray preparation, hybridization of reverse transcribed mRNA, and acquisition of microarray images as described in Methods and Materials.

Given this fact that all transcriptome data would not been obtained immediately, another work of mine was to address the issue whether or not FZB42 is able to colonize plant roots and, if so, how its colonization patterns on various plants are. *B. amyloliquefaciens* FZB42 would be labeled and expected to be recovered directly from plant roots and used for transcriptomic investigation. Moreover, FZB42 wild type and mutants may be labeled with different fluorescent colors and then separately recovered, e.g., by FACS, to compare their transcriptomic response. This is a method which should depict a less distorted picture of how the bacteria regulate their gene expression in the cross-talk with plants, compared with the method of using root exudates. However, a big challenge underlying this method is to collect enough bacterial cells for RNA preparation. Accordingly, using root exudates for the transcriptome investigation was kept to be a substitute method in case that the practice on the idea failed.

In summary, this doctoral work began with labeling *B. amyloliquefaciens* FZB42 with GFP and then observing its colonization on three different plants genera from monocotyledonous maize, dicotyledonous *Arabidopsis* to aquatic duckweed *Lemna* in a gnotobiotic system, respectively. Simultaneously, FZB42 wild type and seven derivative mutants were tested for their transcriptomic responses to maize root exudates using DNA microarray. This work will provide a first insight into which genes of G^+ PGPR specifically expressed in response to plant root exudates, and what molecular mechanisms are underlying these responses, helping us to understand the major behaviors of FZB42 in plant-microbial interactions.

2 Materials and methods

2.1 Chemicals and materials

All chemicals and materials used in the present study are listed in table 1.

Table 1: Chemicals and materials used in this work

Manufacturer	Product
Amersham Pharmacia	[γ-^{32}P]ATP, Plus One Tris-Base, Plus One EDTA, Plus One boric acid
Bioron	Taq polymerase
Fermentas	DNA markers, dNTPs, restriction endonucleases, RiboLock ribonuclease inhibitor (40U/ µl), T4 DNA ligase, T4 kinase, T4 Polynucleotide kinase, Lambda DNA/ EcoRI+HindIII Marker, O'GeneRuler™ Ultra Low Range DNA Ladder, pUC19 DNA/MspI (HpaII) marker
Fluka	$CaCl_2$, EDTA
Macherey-Nagel	Nitrocellulose membrane porablot NCL, Nucleo Spin ® Extract II, Nucleo Spin RNA L
Merck	β-Mercaptoethanol, Ethanol (reinst) 96 %
MP Biomedicals	Urea pure
Promega	pGEM-T® Vector systems
Qiagen	QIAEX II gel extraction kit, QIAprep Spin mini prep kit, QIAquick PCR purification kit
Roche	Anti-DIG AP, Ampicillin, blocking reagent, DIG-dUTP, kanamycin
Roth	Agarose, chloramphenicol, citric acid, DEPC, $FeCl_2$,$FeCl_3$, $Fe_2(SO_4)_3$, formaldehyde, L-glutamic acid, glycerol, HEPES, IPTG, K_2HPO_4, H_2KPO_4, $MgSO_4$, $MnCl_2$, $MnSO_4$,Na-acetate, Na-citrate, Na_2CO_3,

	$(NH_4)_2SO_4$, peptone, SDS, Proteinase K, Rotiphorese Gel 40 (19:1), Rotiphorese Gel 40 (29:1), TEMED, Tris, Triton-X 100, Tween 20, XGal, yeast extract, $ZnCl_2$, QIAquick Hybridization Buffer, Phenol, Saure Phenol, pH 4
Serva	Agar, APS, boric acid, casamino acids, DTT, EGTA, erythromycin, glucose, N-Lauroylsarcosine-sodium, lincomycin/HCl, $MgCl_2$, MOPS, NaN_3, Na_2SO_4, ONPG, L-tryptophan
Sigma	Oligonucleotides, Murashige and skoog basal salt mixture
USB	Low-melting point agarose, Thermo Sequenase cycle Sequencing kit

2.2 Plasmids, bacterial strains and primers

The plasmids, bacterial strains and primers used in this study are listed in tables 2, 3, and 4 respectively.

Table 2: Plasmids used in this work

Plasmid/origin	Description
pGEM-T/Promega	Cloning vector, Ap^r
pECE73/BGSC	$Cm^r \rightarrow Km^r$ exchange vector, Ap^r
pECE149/BGSC[Kaltwasser *et al*. 2002]	Integration vector obtained from BGSC, carrying a *gfp*+ gene, Ap^r
pECE150/BGSC	Integration vector obtained from BGSC, carrying a *cfp* gene, Ap^r
pECE163/BGSC	Integration vector obtained from BGSC, carrying a *dsRed* gene, Ap^r
ptdTomato-N1/Clontech	Mammalian expression vector carrying a *tdTomato* gene, Km^r
pVBF [a]	Integrative vector carrying Em^r cassette flanked by neighbouring sequences of *amyE*; pUC18 derivative

pFB01 [a]	Integrative vector carrying *Em*^{r} and *gfp*+ cassette flanked by neighbouring sequences of *amyE*; pVBF derivative; used for FB01
pFB02 [a]	Integrative vector carrying *Em*^{r} and *gfp*+ cassette flanked by neighbouring sequences of *amyE*; pVBF derivative
pFB03 [a]	Integrative vector carrying *Em*^{r} and *dsRed* cassette flanked by neighbouring sequences of *amyE*; pVBF derivative; used for FB03
pFB04 [a]	Integrative vector carrying *Em*^{r} and *tdTomato* cassette flanked by neighbouring sequences of *amyE*; pVBF derivative; used for FB04
pFB05 [a]	Integrative vector carrying *Spc*^{r} and *tdTomato* cassette flanked by neighbouring sequences of *amyE*; pVBF derivative; used for FB05
pFB06 [a]	Integrative vector carrying *Spc*^{r} cassette flanked by neighbouring sequences of *ydbM*; pGEM-T derivative; used for FB0612 and FB0614
pFB07 [a]	Integrative vector carrying *Spc*^{r} cassette flanked by neighbouring sequences of *bcd*; pGEM-T derivative; used for FB0712 and FB0714
pFB11 [a]	Integrative vector carrying *Spc*^{r} cassette flanked by neighbouring sequences of *iolA*; pGEM-T derivative; used for FB1112 and FB1114

[a] The plasmids were constructed in this work.

Table 3: Bacterial strains used in the present study

Strain	Genotype	Reference
E. coli DH5α	*supE44* Δ*lacU169* (*Φ80 lacZΔM15*) *hsdR17 recA1 gyrA96 thi-1 relA1*	Laboratory stock

E. coli JM101	*supE thiA (lac-proAB) tra D36, pro AB , lac 9,Z A M15*	Laboratory stock
B. amyloliquefaciens FZB42	Wild type	FZB Berlin
B. subtilis 168	*trpC2*	Laboratory stock
B. subtilis FZB37	Wild type	FZB Berlin
FB01	FZB42 *amyE*::*Em* ^{r}-gfp+	This study
FB01mut	FB01 with an unknown spontaneous mutation	This study
FB02	FZB42 Δ*pabB*::*Km* ^{r} *amyE*::*Em* ^{r}-gfp+	This study
FB03	FZB42 *amyE*::*Em* ^{r}-dsRed	This study
FB04	FZB42 *amyE*::*Em* ^{r}-tdTomato	This study
FB05	FZB42 *amyE*::*spc* ^{r}-tdTomato	This study
FB0612	CH12 *ydbM*::*spc* ^{r}	This study
FB0614	CH14 *ydbM*::*spc* ^{r}	This study
FB0712	CH12 *bcd*::*spc* ^{r}	This study
FB0714	CH14 *bcd*::*spc* ^{r}	This study
FB1112	CH12 *iolA*::*spc* ^{r}	This study
FB1114	CH14 *iolA*::*spc* ^{r}	This study
CH12	*dpks2KS1*::*cat*, Δ*pks3KS1*::*ermAM*, no synthesis of macrolactin and difficidin	X. -H.Chen
CH14	*dpks1KS1*::*cat*, Δ*pks2KS1*::*neo*, no synthesis of macrolactin and bacillaene	X. -H.Chen
CH30	FZB42 *sigV*::*Em* ^{r}	X.-H.Chen
CH33	FZB42 *sigB*::*Em* ^{r}	X.-H.Chen

TF1	FZB42 *degU*::*Em* ^{r}	T.-F.Huang
UL1	FZB42 *sigX*::*Em* ^{r}	U. Leppert
AM05	FZB42 *sigD*:: *spc* ^{r}	A. Mariappan
AM06	FZB42 *sigM*:: *spc* ^{r}	A. Mariappan
AM07	FZB42 *abrB*:: *spc* ^{r}	A. Mariappan

Table 4: Primers or oligonucleotides used in this study

Primer name	Sequence (5' to 3' end)		Use
amyBack-1	AGCGAAATTACCTGACGGCAG	21	FB01
amyBack-2	AGCTCAAGTTCCGTCACACCTG	22	FB01
amyFront -1	AGTTTGACGTCTCTCCGATTTCGCCGACAACAC	33	FB01
amyFront-2	TCGATTTGTTTGCAGTTTCAGCG	23	FB01
Tomato up	GATAATGGTACCAATGGTGAGCAAGGGCG	29	FB04
Tomato dw	TCCATTAACTAGTCTTACTTGTACAGCTC	29	FB04
iolA_frN	ATCGTCTCATCAATCGAGCGGT	22	
iolA_revN	AGGAGGCAATGAGAATGGCAGAG	23	
Bcd_fr3	GCCCGTCAGGACGATAATGTCTA	23	
Bcd_rev3-1	TCTTGGTTCCTTCAATCGAGGCC	22	
Bcd_rev3-2	GGTTAATCCGAAAATGGAGGCGA	23	
ydbM_fr	TGTTGTGTTCTTCTGTATTCCGA	22	
ydbM_rev	CTCAGATCATCAGTTGAAGGACG	23	
baeI1_fr	CACTTGGTGACGCCGTTTC	19	RT-
bcd1_fr	ATTGAGCGGGTGCTCGATAT	20	RT-
dfnJ1_fr	GTCGGCATGGGAGAGGAA	18	RT-
glvA1_fr	CGGATGATATGGTGAAAAAATCAA	24	RT-
hag1_fr	GCTGAGGGTGCATTAAACGAA	21	RT-
iolA1_fr	AGCGCGTGCAAGCGTTA	17	RT-
iolD1_fr	AGCAGGTGGAGCAGGAATACA	21	RT-
ptb1_fr	GGGAACCCTATGCCGAAAG	19	RT-
sigW1_fr	AGCAGAAGGGCTGACGATGT	20	RT-
ydbM1_fr	GCCTGAACGGACCGATTAAA	20	RT-

yfjT1_fr	GACCCTGAATCAACGGACGTT	21	RT-
baeI2_rev	CGTGCATGATTAACTCCTTCTCA	23	RT-
bcd2_rev	CAGACGGTCAGCCGCTAAGT	20	RT-
dfnJ2_rev	GGGCCGGTTTATGATAGACTTG	22	RT-
glvA2_rev	TTCCCGCCCTTCCATGA	17	RT-
hag2_rev	CGTTAGCCGCTTGTGTAGCA	20	RT-
iolA2_rev	CTTCAAGGTGGGCGTCATTT	20	RT-
iolD2_rev	GCGGGACACGGGCTTTA	17	RT-
ptb2_rev	CGCCTCCATTTTCGGATTAA	20	RT-
sigW2_rev	ACGGCGTCTTCAGGGAGAA	19	RT-
ydbM2_rev	GCTCCATTTCCCCGATACG	19	RT-
yfjT2_rev	GACCCTGAATCAACGGACGTT	21	RT-
#01_Igr3849	GAGAGCTGATGGCCGGTGAAAATCA	25	N.B.
#02_Igr3873	GCCTTCTGTAAAATAAGAAGGATTCCCACT	30	N.B.
#03_Igr3893	GATGTTTTACCAAATTATAAAGTGCGTACA	30	N.B.
#04_Igr3906	ACCACAAGGGGAGCATTAAAGCTGAGA	27	N.B.
#05_Igr3925	CCCCTCCTCGGGATGTCCATCATTC	25	N.B.
#06_Igr3927	AACCCCTTCATCCAAGGAGCCAATTTTG	28	N.B.
#07_Igr3931-1	CCGCTTCTCACCTGATTGACACATT	25	N.B.
#08_Igr3931-2	TTGCCTGCAGAATGCAGTCAACAAG	25	N.B.
#09_Igr3959	TGAAAAGGAGGACATCAGGTCAAGATAAGG	30	N.B.
#10_Igr4023	AGGTTTTCGCGGTGCCACCTTTATTAA	27	N.B.
#11_Igr4026	TCATATGGTATGTATTTCAACCCCACGATA	30	N.B.
#12_Igr4028	GCACATACGGGACTAAACAATGGGGAA	27	N.B.
#01c_Igr3849	TGATTTTCACCGGCCATCAGCTCTC	25	N.B.
#02c_Igr3873	AGTGGGAATCCTTCTTATTTTACAGAAGGC	30	N.B.
#03c_Igr3893	TGTACGCACTTTATAATTTGGTAAAACATC	30	N.B.
#04c_Igr3906	TCTCAGCTTTAATGCTCCCCTTGTGGT	27	N.B.
#05c_Igr3925	GAATGATGGACATCCCGAGGAGGGG	25	N.B.
#06c_Igr3927	CAAAATTGGCTCCTTGGATGAAGGGGTT	28	N.B..
#07c_Igr3931-1	AATGTGTCAATCAGGTGAGAAGCGG	25	N.B.
#08c_Igr3931-2	CTTGTTGACTGCATTCTGCAGGCAA	25	N.B.
#09c_Igr3959	CCTTATCTTGACCTGATGTCCTCCTTTTCA	30	N.B.
#10c_Igr4023	TTAATAAAGGTGGCACCGCGAAAACCT	27	N.B.

#11c_Igr4026	TATCGTGGGGTTGAAATACATACCATATGA	30	N.B.
#12c_Igr4028	TTCCCCATTGTTTAGTCCCGTATGTGC	27	N.B.
Nr.2c_54.5	CTTTTCGTAATCTCTGTTCTGCTGATC	27	N.B.
Nr.9c_53.6	AATCCAAATTCTTACCCTTATCTTGACC	28	N.B.
Nr.11c_56.7	GGGGCTTATCGTGGGGTTGAAATA	24	N.B.
Nr.9c_new_52.9	CATGTTAAACAAATTTTGCTAACGAATC	28	N.B.
5S-N2	TGAAGAGCTTAACTTCCGTGTTCGGCAT	28	N.B.
IgrA_3817	GAGAGGTCCTAACCCTTTAAGTA	23	N.B.
IgrB_3839	AGCTAGCTTGATATTTCGTCATTC	24	N.B.
IgrC_3941	GGTTGTAGCATTGGTGCTACAT	22	N.B.
IgrD_3947	GGGCTCCCAAATCAAAAAAATGTT	24	N.B.
IgrE_3940	GAATGACGAAATATCAAGCTAGCT	24	N.B.

RT-PCR: Real time PCR; N.B.: Northern Blot; P.E.: primer extension. The enzyme recognition site within each primer is underlined.

2.3 Media, buffers and solutions

All media used in this work (Table 5) were prepared and sterilized according to [Cutting *et al*. 1990; Sambrook *et al*. 2001]. Antibiotics and other supplementary compounds are listed in Table 6.

Table 5: Media, buffer and solutions used in this work

Medium	ingredients
Luria Broth	1% w/v peptone, 0.5% w/v yeast extract, 0.5% w/v NaCL
Murashige-Skoog medium	4.3 g/l basal salt mixture (sigma) supplemented with 0%, 1% or 3% sucrose.
Steinberg Medium	KNO_3 350mg/l, KH_2PO_4 90 mg/l, K_2HPO_4 12 mg/l, $MgSO_4 \cdot 7H_2O$ 100 mg/l, $Ca(NO_3)_2 \cdot 4H_2O$ 295 mg/l, $MnCl_2 \cdot 4H_2O$ 0.18 mg/l, H_3BO_3 0.12 mg/l, Na_2MoO_4 0.044 mg/l, $ZnSO_4 \cdot 7H_2O$ 0.18 mg/l, $FeCl_3 \cdot 6H_2O$ 0.76 mg/l,

	$Na_2EDTA \cdot 2H_2O$ 1.5 mg/l
5× 1C medium	3.5% w/v pancreatic digest of casein, 1.5% w/v papain digest of soya flour, 2.5% w/v NaCl
1CS Medium	1× 1C medium, 10% v/v soil extract, 0.25mg/ml root exudates, 0.1% glucose.
Landy Medium	Glucose 2.00%, glutamate 0.50%, $MgSO_4$ 0.05%, KCl 0.05%, KH_2PO_4 0.10%, $FeSO_4 \cdot 7H_2O$ 0.015%, $MnSO_4$ 0.50%, $CuSO_4 \cdot 5H_2O$ 0.02%, yeast extract 0.01%
Electrophoresis	
TAE-Buffer	40 mM Tris, 1.1 ml/l acetate acid, 1 mM EDTA, 0.5 µg/l Ethidium bromide
10 × TBE	890 mM Tris, 890 mM Boric acid, 20 mM EDTA
10 × MEN	200 mM MOPS, 50 mM Na-Acetate, 10 mM EDTA
DAN Agarose gel	0.8% agrose in 1 × TAE
RNA-Agarose gel	1 % und 1.5 % in 1× MEN-Buffer, 5.6 % Formaldehyde
Urea-Acrylamid gel	6 % AA/BAA (19:1), 1× TBE, 7M Urea, 0.08 % APS, 0.01 % TEMED
Cell manipulation	
Killing Buffer	20 mM Tris-HCl (pH 7.5), 5mM $MgCl_2$, 20mM NaN_3
Lysis Buffer	20 mM Tris-HCl (pH 8.0), 50 mM NaCl, 10 mM EDTA, 4 mg/ml Lysozyme (fresh prepared)
Resuspension Buffer	10 mM Tris-HCl (pH 8.0), 150 mM NaCl, 10 mM EDTA
Transformation Buffer	1 × SSM, 1 mM EGTA, 0.5 % Glucose, 20 mM $MgCl_2$
MDCH Buffer	1×PC, Glucose 1%, L-Trp 0.05mg/ml, $FeCl_3$/Na-Citrate 0.1mg/ml, $MgSO_4$ 3mM,

	Casein hydrolysate 0.1%, Na-Glutamate 2.5mg/ml
MD Buffer	1×PC, Glucose 1%, L-Trp 0.05mg/ml, $FeCl_3$/Na-Citrate 0.1mg/ml, $MgSO_4$ 3mM,
Northern Blot	
P1-Dig-Buffer	100 mM Maleic acid (pH 7.5), 150 mM NaCl
P2-Dig-Buffer	Blocking reagent in P1-Buffer
AP-Buffer	1 % Blocking reagent in P1-Buffer
20× SSC	175 g/ l NaCl, 88.2 g/ l Na-Citrate Dihydrate
10 × TBST	100 mM Tris pH 8.0, 1.5 M NaCl, 0.5% Tween 20
Tris-HCl Buffer	1 M K_2HPO_4 / KH_2PO_4 pH 7.0
10 × TE	100 mM Tris-HCl (pH 7.5), 10 mM EDTA
Loading Buffer	
6 × DNA Loading Buffer	30 % Glycerine, 10 mM EDTA (pH 8), 0.25 % Bromphenol blue
Stop Solution	95 % deionized Formamide, 20 mM EDTA (pH 8), 0.05 % Bromphenol blue, 0.05 % Xylen cyanol
1.6 × RNA Loading Buffer	0.75 × MEN, 28.5 % deionized Formamide, 3 % Formaldehyde, 16 µg/ml Ethidium bromide
2 × RPA Buffer	98 % deionized Formamide, 1 mM EDTA (pH 8), 0.1 % Bromphenol blue, 0.1 % Xylen cyanol
Solution	
10 × SMM	20 g/ l $(NH_4)_2SO_4$, 140 g/ l K_2HPO_4, 60 g/l KH_2PO_4, 10 g/ l Na-Citrate-Dihydrate
10 × PC	0.8M K_2HPO_4, 0.45M H_2KPO_4, Na·Citrate, pH 7.0

Soil Extract	Mix 500g soil with 1 litre distilled water, filter the supernatant and then autoclave, store at -4°C until use.

Table 6: Antibiotics and Supplements

Supplement	Final concentration
Ampicillin	100 µg/ml
Chloramphenicol	20 µg/ml (for *E. coli*), 5 µg/ml (for *Bacilli*)
Erythromycin	1 µg/ml (for *Bacilli*)
Kanamycin	20 µg/ml (for *E. coli*), 5 µg/ml (for *Bacilli*)
Lincomycin	25 µg/ml (for *Bacilli*)
Spectinomycin	100 µg/ml (for both *E. coli* and *Bacilli*)
X-Gal	40 µg/ml
IPTG	0.2 mM

2.4 Investigation of plant colonization by FZB42

2.4.1 Growth conditions of bacterial strains and plant materials

Bacterial strain *Bacillus amyloliquefaciens* FZB42 and *Bacillus subtilis* 168 were cultivated routinely in Luria broth (LB) at 28°C. FZB42 was deposited as strain 10A6 in the culture collection of *Bacillus* Genetic Stock Center (BGSC). *Zea mays* seeds were obtained from company Saaten-Union, Germany. The seeds of *Arabidopsis thaliana* ecotype Columbia-0 were obtained from AG genetics, Department of Biology, Humboldt University, Berlin. The duckweed clone L. minor ST was a courtesy from Institute of General Botany and Plant Physiology, Friedrich-Schiller-University, Jena, Germany. L. minor ST was propagated axenically in filter-sterilized Steinberg medium as described previously [Idris *et al*. 2007].

2.4.2 Construction of fluorescent protein-labeled FZB42

2.4.2.1 GFP-labeling of FZB42

The upstream sequence of *amyE* gene of FZB42 (amy-up) was amplified from FZB42 chromosomal DNA using primers amyFront-1 and amyFront-2. The downstream sequence of *amyE* gene (amy-dw) was amplified with primers amyBack-1 and amyBack-2. Amy-up and amy-dw were respectively inserted into vector plasmid pUC18Emr, yielding a recombinant plasmid pVBF. The *gfp*+ gene together with an upstream located P_{spac} promoter element was derived from plasmid pECE149 (BGSC) [Oliver *et al*. 2000; Kaltwasser *et al*. 2002] and cloned into plasmid pVBF. The resulting integrative plasmid pFB01 containing *gfp*+ flanked by two *amy*E border sequences. (Figure 2, Panel A) was transformed into competent FZB42 cells as described previously [Koumoutsi *et al*. 2004]. The *amyE*$^-$ transformants were selected onto LB plates supplemented with 1% starch, 1µg/ml erythromycin and 25µg/ml lincomycin. Homologous recombination was confirmed by PCR and fluorescence microscopy.

2.4.2.2 Red fluorescent protein-labeling of FZB42

Plasmid pECE163 (BGSC) containing the DsRed gene without promoter was linearized by endonuclease *EcoR*I and then blunted by Klenow Fragment. The DsRed gene cassette was subsequently isolated from pECE163 using the second restriction enzyme *Spe*I and cloned into plasmid pFB01 where the *gfp*+ gene had been removed by *Kpn*I and *Spe*I, leaving the P_{spac} promoter and the *trp* terminator intact. The cohesive ends of the "empty" pFB01 created by *Kpn*I were also blunted by Klenow Fragment and then ligated with the DsRed fragment derived from pECE163. The new recombinant plasmid yielded after ligation was named pFB03.

Vector pTdTomato was obtained from Roger Tsien [Shaner *et al*. 2004] and the TdTomato gene was amplified with a forward primer "Tomato_up" and a reverse primer "Tomato_down". The amplified PCR product was digested by *Kpn*I and *Spe*I and then cloned into the "empty" plasmid pFB01 lacking *gfp*+ while still containing the preceding P_{spac} promoter and the terminator as described above, thus resulting a new plasmid pFB04.

The two plasmids (pFB03 and pFB04) with red fluorescence protein (RFP) gene were respectively transformed into FZB42 as described above. The yielding transformants were

also similarly screened as above, obtaining strains FB03 with DsRed and FB04 with TdTomato, respectively.

2.4.2.3 Comparison of fluorescence intensity

To compare the fluorescence intensities of strain FB01 and the spontaneous mutant FB01mut, fresh bacterial cultures were grown in LB media at 37°C until OD_{600} reached ~2.4. The samples for fluorescence measurements were prepared by dissolving the bacterial pellets obtained after centrifuge with cell fixation buffer (1×PBS with 0.3% Formaldehyde) and then diluting with the same buffer to an OD_{600} of 0.2. The diluted cells of 200µl in Costar 96 black clear bottom plates (Corning Life Sciences) were analyzed by SpectraMax M2e (Molecular Device). The relative fluorescence intensity was measured at excitation values set at 485nm and emission values set at 520nm.

2.4.2.4 Test of fluorescence stability of gfp-labeled FZB42

GFP-labeled FZB42 stains were grown in LB medium in the absence of antibiotic for successive 4 days, resulting in at least 50 generations. Approximately every 12 hours the cells were inoculated into a fresh medium with 1:1000 dilutions. GFP stability was evaluated by examining the fluorescence of the colonies onto LB agar plates obtained by serial dilution. Over 400 colonies of each of FB01 and FB01mut were examined for the occurrence of fluorescence.

2.4.3 Colonization of plants by FZB42

2.4.3.1 Colonization of maize seedling roots

i) Surface sterilization of maize seeds: Maize seeds were treated with 70% ethanol for three minutes and then with 5% (v/v) sodium hypochlorite for another three minutes before a final rinse of 5 times with sterile distilled water.

ii) Maize seedlings: After surface sterilization eight maize corn kernels, embryo upside, were placed in a standard 9 cm Petri dish filled with seven ml sterile water (1/2 distilled water+1/2 tap water) and then incubated in dark at 30°C for overnight. In the second morning 250µl water was taken from the Petri dish and spread onto a LB plate in order to check contamination. The seeds were continued to incubate with refreshed water in the

same condition until they germinated after 40-45 hours. The germinated corns with a root of approximate 2cm were chosen for next steps.

iii) Inoculation and incubation: Bacteria were grown in Luria Broth till OD_{600} reached 1.0. The cultures were diluted with fresh LB by 1000 times (~10^5CFU) and then shaken at 37°C for another 15 minutes before being used for inoculation. The roots of the maize seedlings described above were inoculated by dipping into the culture, softly swirling, for two minutes. Finally the inoculated maize were grown in soft agar (0.8%) containing basal Murashige-Skoog medium (without sucrose) and incubated in plant growth room (24°C, 16 hours daytime, 8 hours dark time).

2.4.3.2 Colonization of *Arabidopsis* roots

The seeds of *Arabidopsis thaliana* ecotype Columbia-0 were similarly surface-sterilized as above with reduced treatment time of merging into 70% ethanol for only 30 seconds. The sterilized seeds were germinated on an agar (0.6%) plate of basal Murashige-Skoog medium containing 1% sterile sucrose and grown at 24°C for 7 days. The seedlings were likewise inoculated as described for maize seedlings and subsequently mounted onto another square agar (0.8%) plate (12cm×12cm) of basal Murashige-Skoog medium. The plate was kept inclined, standing 30°C to the vertical, and incubated in the same condition as for maize seedlings.

2.4.3.3 Colonization of *Lemna minor*

Lemna minor ST was grown as previously described [Idris *et al*. 2004] with minor modification. Briefly, one sterile *Lemna* plantlet bearing two fronds were transferred into a well of a micro-titer. Each well of 16 mm in diameter contained 2 ml Steinberg medium and was inoculated with 0.2% bacterial culture of OD_{600}=1.0. The micro-titer plates were incubated at 20°C in a growth chamber with 12-hour light and 12-hour dark time. Every two day the media were refreshed by pipetting out the old media softly and refilling with new ones.

2.4.4 Specimen preparation for microscopy

The roots of maize and *Arabidopsis* of seven days after planting were sampled for microscopy. In terms of *Lemna*, both roots and fronds of 1 day, 5 days and 9 days,

respectively, after inoculation were observed. While *Lemna* and *Arabidopsis* roots could be observed directly with microscope, maize specimens were prepared by scratching a piece of root surface, around 1cm in length, from different parts of a root with a sterile blade or by cutting a cross section of 50 μm in thickness with a microtome. All specimens were softly rinsed with sterile distilled water prior to merging into saline for microscopic observation.

For electron microscopy, a 10 mm primary root segment of maize seedlings of 7 days old was taken 25 mm below the kernels, removing the lateral roots. The segment was also softly rinsed with sterile distilled water and then divided into two 5 mm segments and processed for TEM and SEM respectively. *Lemna* of 9 day old were sampled for imaging both ventral surfaces of fronds and roots by SEM.

2.4.5 Microscopy

2.4.5.1 Fluorescent microscopy

In many cases samples were firstly examined with an epifluorescence microscope Zeiss Axiophot XIOPHOT. GFP fluorescence was examined using a filter set of 450-490nm excitation filter and LP520 emission filter, while red fluorescence was viewed by using a BP546 excitation filter and a LP590 emission filter.

2.4.5.2 Confocal laser scanning microscopy

Confocal Laser Scanning Microscopy (CLSM) was performed with a Leica DM IRE2&DM IRB system. While GFP fluorescence was recorded by using an excitation laser of 488 nm (Argon laser) and collecting the emission of 500-550 nm, an excitation with NeHe laser of 543 nm was used and the emission band of 575-655 nm was collected for DsRed/TdTomato fluorescence. Transmission light was collected to visualize root structure and was designate as red color in later image reconstruction in order to manifest the contrast with green color. Images were acquired and reconstructed by Leica Confocal Software (LCS 2.6).

2.4.5.3 Transmission electron microscopy

Samples were fixed with 2.5% glutaraldehyde in 0.1 M Na-cacodylate buffer (pH7.4) for 24 h at 4°C. Afterward the samples were rinsed three times for 1 h with the old 0.1 M

Na-cacodylate buffer of 4°C. A second fixation was performed for 5 h at 4°C by using Na-cacodylate buffer containing 2% osmium tetroxide. The specimen were subsequently rinsed in cold Na-cacodylate buffer solution, poststained with 1% uranyl acetate in 0.05 M maleate buffer solution (pH5.2) for 5h at 4°C, dehydrated in a graded ethanol series, infiltrated and embedded in Spurr's epoxy resin and polymerized for 24 h at 70°C. Ultruthin-sections were cut and transferred to uncoated 300 mesh thin-bar-grids, stained with uranyl and Reynold's lead citrate and viewed with a Zeiss EM 900 electron microscope (Carl Zeiss AG, Oberkochen, Germany).

2.4.5.4 Scanning electron microscopy

For scanning electron microscopy, samples were fixed with glutaraldehyde as described above. After rinsing several times in Na-cacodylate buffer solution specimens were postfixed for 4.5 h in 1% osmium tetroxide at 4°C and washed again in Na-cacodylate buffer solution. Dehydration through a graded series of ethanol solutions and finally 100% acetone was followed by critical point drying with liquid CO_2 using the CPD 030 (BAL-TEC, Germany). The specimens were then mounted on stubs, sputtered with gold (Sputter Coater SCD, 005, BAL-TEC, Germany) and examined with a LEO 1430 scanning electron microscope.

2.5 Transcriptomic investigation of FZB42 to root exudates

2.5.1 Root exudates

The maize seeds used here were the same as those in plant colonization experiments. Root exudates were collected from the maize seedlings grown in gnotobiotic (axenic) system comprising only autoclaved water (1/2 distilled water + 1/2 tape water, v/v). Forty germinated seeds after surface sterilization were transferred into test tubes filled with 2 ml autoclaved water, keeping maize corns just above the surface of water. The system (as shown in Figure 1) for maize growing were kept in a sterile condition and maintained for 8 days at 24°C in a 16-hour light/8-hour dark plant growth room. In the first two days adequate water was supplemented to the tubes every day, each time pulling the seedlings up to a higher position to keep the maize corns always above water surface as the roots extended. From the third day the water containing exudates began to be collected, afterwards refilling the tube with new sterile water. The collection was repeated every day

until the eighth day after transferring. The collected exudates were pooled together and stored at -80°C until lyophylization. The lyophilized exudates were measured for the dry weight and then dissolved in the specific amount of water. After centrifuge supernatant was filtered to prepare sterile exudate solution, while small amount of insoluble pellets were dried and deduced from the dry weight of crude exudates. The prepared exudate solution was adjusted to a proper concentration and stored at -80°C in dark until use.

Figure 1: The gnotobiotic system used for collection of maize root exudates. The maize seedlings were grown in sterile water, keeping the corns just above the surfaces of water. The seedlings shown here were in the sixth day after being transferred into test tubes.

2.5.2 Standard molecular biology methods

DNA manipulations, such as digestion with restriction endonucleases and ligation, were performed according to the instructions supplied by the manufacturer. Agarose-gel-electrophoresis, fluorescent visualization of DNA with ethidium bromide, spectrophotometric quantification of DNA/RNA as well as preparation of $CaCl_2$-competent *E. coli* cells followed by transformation of plasmid DNA were carried out with standard procedures described previously [Sambrook *et al*. 2001]. Bacterial chromosomal DNA from *Bacilli* was prepared as previously described [Cutting *et al*. 1990]. Polymerase chain reaction (PCR) was done using the GeneAmp PCR system 2700 (Applied Biosciences). Ligation of PCR products to pGEM-T vector was carried out following the instructions of the manufacturer (Promega). Plasmid DNA isolation and recovery of DNA from agarose

gels were performed with QIAprep Spin mini prep kit and QIAEX II gel extraction kit, respectively.

2.5.3 Transformation in *Bacillus amyloliquefaciens*

Competent cells of *B. amyloliquefaciens* FZB42 were obtained by modifying the two-step protocol previously published [Kunst *et al*. 1995]. Cells were grown overnight in LB medium at 28°C (180 rpm). The next day the cells were diluted in MDCH buffer to an OD_{600} of 0.3. The cell culture was then incubated at 37°C under vigorous shaking (210 rpm) until the middle of exponential growth (OD_{600} 1.2~1.4). Dilution with an equal volume of MD medium was followed and the cells were further incubated under the same conditions for 1 hour. Further on, 8 ml culture was transferred to a sterile falcon tube and centrifuged at 5,000 rpm for 3 minutes (room temperature). The pellets were resuspended in 200 µl of the supernatant and the desired DNA (1 µg) with 2 ml transformation buffer was added to them. After incubation at 37°C for 20 minutes with an intermittent shaking, 1 ml LB medium containing sublethal concentration (0.1µg/ml) of the appropriate antibiotic was added. The cells were grown under vigorous shaking for 90 minutes and then plated on selective agar plates.

2.5.4 Design of *B. amyloliquefaciens* microarray

The Bam4kOLI microarray used in this study was based on the sequenced genome of *B. amyloliquefaciens* FZB42 [Chen *et al*. 2007]. The array contained 3931 50-70mer oligonucleotides representing predicted protein-encoding genes and small non-coding RNA genes of FZB42. In addition, the array included stringency controls with 71%, 80% and 89% identity to the native sequences of five genes, *dnaA*, *rpsL*, *rpsO*, *rpsP*, and *rpmI*, to monitor the extent of cross hybridization. The array also contained alien DNA oligonucleotides for 4 antibiotic resistance genes (Em^r, Cm^r, Nm^r and Spc^r) and 8 spiking controls as well as 1 empty control (nothing spotted). All oligonucleotide probes were printed in four replicates. Microarrays were produced and processed as described previously [Brune *et al*. 2006].

Oligonucleotides were designed using the Oligo Designer software (Bioinformatics Resource Facility, CeBiTec, Bielefeld University). Melting temperature of the oligonucleotides were calculated based on %GC and oligo length, ranging from 73°C to

83°C (optimal 78 °C). Salt concentration was set to be 0.1 M. QGramMatch was used to analyze uniqueness of the oligos.

2.5.5 Total RNA preparation

One fresh colony of *B. amyloliquefaciens* FZB42 was inoculated into 1C medium containing 0.1% glucose and shaken at 210 rpm and 24°C. After 14 hours the obtained preculture was used to inoculate a new 1C medium supplemented with 10% soil extract and 0.25 mg/ml maize root exudates. The culture was shaken under the same conditions as described for the preculture.

The bacterial cells from exponential phase (OD_{600}=1.0) and stationary phase (OD_{600}=3.0) were harvested for preparation of total RNA. 15 ml of the culture was mixed with 7.5 ml "killing buffer" (stopping mRNA production) and centrifuged at 5,000rpm for 3 minutes at room temperature. The pellet was washed once more with 1 ml "killing buffer" and then immediately frozen in liquid nitrogen. The frozen cell pellets were stored at -80°C until RNA isolation.

Isolation of RNA was performed using the Nucleo Spin RNA L (Macherey Nagel) according to the manufacturer's instructions. In order to avoid possible trace DNA contamination, the isolated RNA was additionally digested with DNase in a solution. After ethanol precipitation RNA pellets were resuspended in 300 µl RNase-free H_2O. The concentration of total RNA was spectrophotometrically determined, whereas its quality was checked on a 1.5% RNA agarose gel under denaturizing conditions (1×MEN, 16% formaldehyde). The samples were mixed with 1.6 volume loading buffer and were incubated at 65°C for 5 minutes prior to loading on the gel. The gel was run in 1×MEN buffer at 60 Volt.

For microarray experiments, at least three RNA samples prepared in three independent experiments were used as biological replicates. In all comparisons dye-swap were carried out to minimize the effect of dye biases.

2.5.6 Synthesis of labeled cDNA, hybridization and image acquisition

Synthesis of first-strand cDNA, microarray hybridization and image acquisition were performed in CeBiTec, the Center for Biotechnology at Bielefeld University. Briefly, Aminoallyl-modified first-strand cDNA was synthesized by reverse transcription according

to DeRisi *et al*. [DeRisi *et al*. 1997] and then coupled with Cy3- and Cy5-*N*-hydroxysuccinimidyl ester dyes (GE Healthcare, Little Chalfont, UK). After hybridization using the HS4800 hybridization station (Tecan Trading AG, Switzerland), slides were scanned with a pixel size of 10 µm using the LS Reloaded microarray scanner (Tecan Trading AG, Switzerland).

2.5.7 Transcriptome data analysis

Transcriptomic data obtained were analyzed by using the EMMA 2.8.2 software [Dondrup *et al*. 2009] developed at the Bioinformatics Resource Facility, CeBiTec, Germany. The mean signal intensity (A_i) was calculated for each spot using the formula $A_i = \log_2(R_iG_i)^{0.5}$ [Dudoit *et al*. 2002]. $R_i = I_{ch1(i)} - Bg_{ch1(i)}$ and $G_i = I_{ch2(i)} - Bg_{ch2(i)}$, where $I_{ch1(i)}$ or $I_{ch2(i)}$ is the intensity of a spot in channel 1 or channel 2, and $Bg_{ch1(i)}$ or $Bg_{ch2(i)}$ is the background intensity of a spot in channel1 or channel 2, respectively. The $\log_2$ value of the ratio of signal intensities (Mi) was calculated for each spot using the formula $M\text{i} = \log_2(R\text{i}/G\text{i})$. Spots were flagged as "empty" if $R \leq 0.5$ in both channels, where R = (signal mean–background mean)/background standard deviation [Serrania *et al*. 2008]. The raw data were normalized by the method of LOWESS (locally weighted scattered plot smoothing). Significant test was performed by the method of false discovery rate (FDR) control and the adjusted p-value defined by FDR was called q-value in this work [Benjamini *et al*. 1995; Roberts *et al*. 2008].

2.5.8 Real-Time PCR

First strands of cDNA were obtained by reverse transcription with RevertAid™ Premium Reverse Transcriptase (Fermentas, St. Leon-Rot, Germany), using random hexamers as primers. Oligonucleotide primers used were designed by software PrimerExpress and listed in Table 4. Real-time PCR was performed with 7500 Fast Real-Time PCR System (Carlsbad, California, USA) and SYBR® Green PCR Master Mix kit (Carlsbad, California, USA), according to the manufacturer's instructions. As a control gene, *gyrA* was used whose expression was not significantly altered in any microarray experiments of this work. Three technical replicates were carried out for each target gene. Quantification was analyzed based on the threshold cycle (Ct) values as described by Pfaffl [Pfaffl 2001].

2.6 Northern blot for small RNA identification

2.6.1 Radioactive labeling of oligonucleotides

Oligonucleotides were radio-labeled at their 5'-OH ends by T4 polynucleotide kinase (T4 PNK) that catalyzes the transfer of γ-phosphate from ^{32}P-ATP. Therefore, 40 pmol of primer were mixed with 4 µl [γ-^{32}P] ATP (10 µCi/ml) and phosphorylation took place by incubation of the mixture with T4-Kinase at 37°C for 30 minutes. The reaction was stopped by heat inactivation at 70°C for 10 minutes.

2.6.2 RNA separation and northern blotting

The total RNA samples of interest used for microarray experiments were separated (5 µg/each sample) on 6% PAA 7M urea gel in 1×TBE buffer. The samples were denatured at 95°C for 5 minutes and then cooled on ice for another 5 minutes. After running the RNAs were then transferred to a positively charged nylon membrane using "Trans-Blot SD Semi-Dry Transfer Cell" (Biorad). Finally the RNAs were immobilized on the membrane by cross-linking using UV radiation.

2.6.3 Hybridization and detection

The membrane was initially incubated in 20 ml QIAquick hybridization buffer for 1 hour at 42°C, while the radioactively-labeled oligo probes were denatured at 95°C for 5 minutes and then immediately cooled on ice to unfold the secondary structures. Subsequently the membrane was hybridized overnight with 1 µl denatured oligo probes at 42°C. The membrane was washed three times at 42°C, each for 15 minutes, with 2× SSC/0.1 % SDS, 1× SSC/0.1 % SDS, and 0.5× SSC/0.1 % SDS, respectively. The results were visualized by FX-ProPhosphorimager (Bio-Rad).

3 Results

3.1 Plant colonization by *B. amyloliquefaciens* FZB42

3.1.1 Fluorescent Protein-labeling of FZB42

The successful labeling of *B. amyloliquefaciens* FZB42 by fluorescent proteins (FPs) was confirmed by PCR, loss of ability to hydrolyze starch and occurrence of green or red fluorescence when the bacterial cells were observed with fluorescence microscope. Since plant colonization studies would be conducted at room temperature, GFP-, DsRed-, and TdTomato-labeled FZB42 cells were grown at 37°C and 24°C, respectively, and their fluorescence was compared. At 37°C, GFP-labeled cells emitted the brightest fluorescence (Figure 2, Panel B) whereas DsRed-labeled cells were the dimmest ones. The fluorescence intensity of GFP-labeled cells showed no apparent difference at two different temperatures, while the brightness of DsRed-labeled cells decreased greatly from at 37°C down to 24°C. In addition, DsRed-labeled bacteria showed a considerable cell-to-cell variation in brightness, probably because the mature time of DsRed at 37°C was as long as around 20 hours [Shaner *et al*. 2005] and even twice longer at room temperature [Bevis *et al*. 2002].

Optimized from DsRed, TdTomato has a faster maturation rate ($t_{0.5}$ for maturation ≈ 1 hours) and a good photo-stability [Shaner *et al*. 2004]. As expected, at 37°C TdTomato-labeled cells (FB04) were much brighter than dsRed-labeled cells (FB03). Nevertheless, FB04 still displayed a detectable cell-to-cell variation in brightness (Figure 2, Panel C&D). Furthermore, like FB03, the brightness of FB04 also decreased significantly at 24°C, possibly because TdTomato was originally developed for labeling mammalian cells grown at 37°C [Shaner *et al*. 2005].

According to these comparisons, GFP-labeled strain FB01 was more suitable than the red fluorescent protein-labeled strains for further colonization studies.

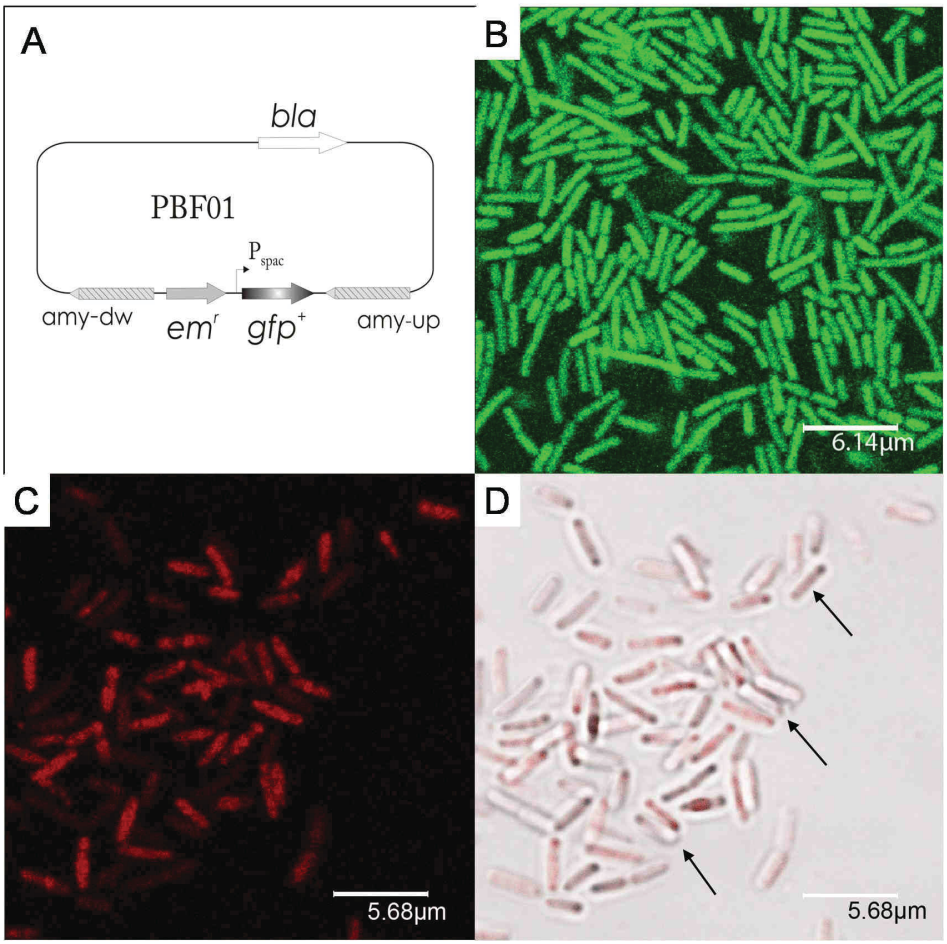


Figure 2: Construction of fluorescent protein-labeled FZB42. Panel A: A schematic map of plamid pFB01 used for constructing GFP-labeled strain FB01, where "amy-up" means the upstream fragment of *amyE* and "amy-dw" means the downstream fragment of *amyE*. Panel B: GFP-labeled FB01mut cells grown at 24°C overnight; Panel C: TdTomato-labeled FB04 cells grown at 37°C overnight; Panel D: an overlay of Panel B and a transmission light image. Note the cell-to-cell variations of fluorescence intensity in Panel D when compared with Panel C. Some cells with obvious lower fluorescent brightness were indicted by arrows in Panel D.

3.1.2 FB01mut, a brighter spontaneous mutant

A spontaneous mutant of FB01 with enhanced fluorescence brightness was occasionally isolated from LB agar. Compared with the parental strain (FB01) under identical growth conditions, the mutant strain (FB01mut) displayed not only a brighter fluorescence (Figure 3, Panel A&B) but also a slightly prolonged resistance to photobleaching. The measurement of fluorescence intensity with microplate readers (see Materials and Methods) suggested that in liquid conditions the fluorescence from FB01mut was at least 1.5 times brighter than that from FB01. No significant difference was found between FB01 and FB01mut in terms of the stability of GFP and the growth rate of two

strains (see 3.1.3). There was no significant difference between the two strains in their ability to colonize a plant like *Lemna minor* ST, either. As a result, FB01mut was finally adopted for plant colonization studies.

A 1500 bp region covering the complete GFP cassette and its flanking promoter and terminator regions (Figure 2, Panel A) was sequenced in order to find possible mutation(s) occurred in FB01mut. Unfortunately, no nucleotide exchange was detected in this region, implying that mutation(s) in other regions might be responsible for the enhanced fluorescence intensity.

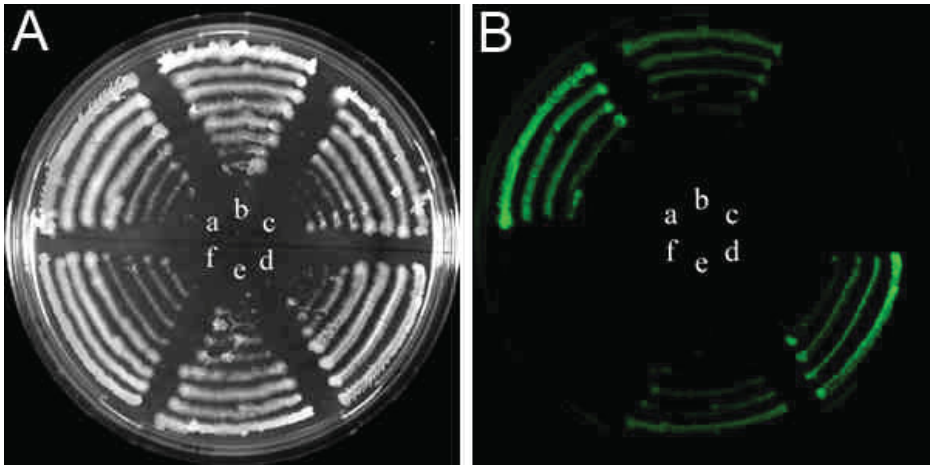


Figure 3: Comparison of the fluorescence intensity of FB01mut, FB01 and FZB42 wild type. After overnight incubation on LB agar at 24°C the strains FB01mut (a&d), FB01 (b&e) and FZB42 (c&f) were visualized with visible light (Panel A) and UV light of 390nm (Panel B) respectively. Note that in Panel B the fluorescence from FB01mut is brighter than that from FB01.

3.1.3 The Stability of GFP and its effect on the growth of FZB42

In order to assess the stability of GFP in strains FB01 and FB01mut, the two strains were successively grown in LB for four days, resulting at least 50 generations, and then examined for fluorescence occurrence. 400 colonies examined for each strain were all able to emit green fluorescence, indicating that GFP can be stably expressed in both strains. At the same time, the three strains FB01, FB01mut, and FZB42 wild type were cultivated in LB medium at 37°C and monitored for their growth. The result showed that GFP expression in FB01 and FB01mut does not have a detectable negative effect on their growth (Figure 4).

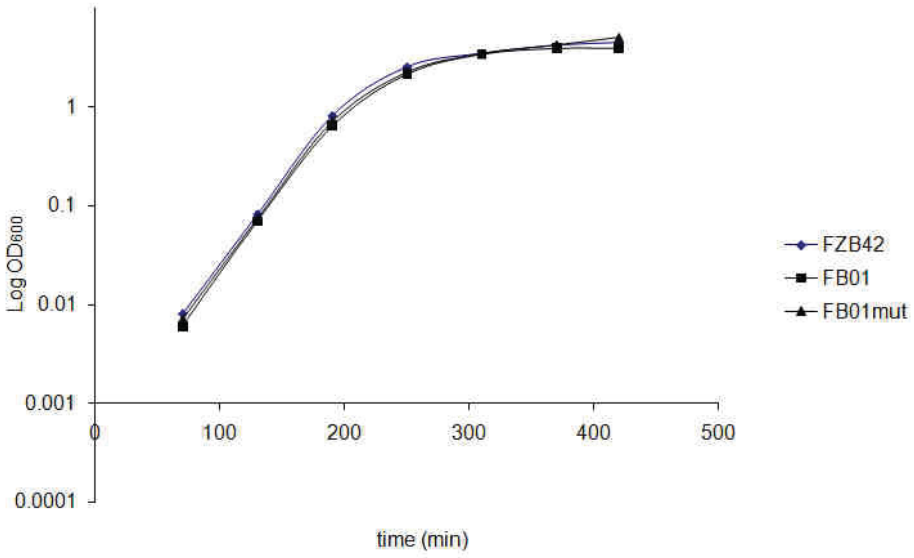


Figure 4: Growth curves in LB of strains *B. amyloliquefaciens* FZB42, FB01 and FB01mut

3.1.4 Colonization of maize seedlings by FZB42

In soft agar of MS basal medium (without sucrose) the primary roots of most maize seedlings could reach approximately 20 cm in 8 days, at an elongation rate of more than 2 cm per day in average. An overall observation of primary roots revealed that the segments within 2--8cm distant from plant basal sites, where a corn kernel remained, were a mostly colonized region by FZB42. On the contrary, few bacterial cells could be observed within the range of 2 cm distant from a root tip. In general, the green fluorescent FZB42 were decreasingly observed from the upper part of a root down to the root tip. Such a descending distribution of FZB42 cells on primary roots was also supported by a numeration experiment (data not shown), although it was difficult to detach all the bacteria from maize roots.

On the highly colonized segments, a number of FZB42 microcolonies could easily been observed around root surfaces (Figure 5, Panel A-D). It is noteworthy that the segments happened to be the regions where abundant lateral roots emerged. However, hardly could fluorescent bacteria be observed on the lateral roots except their bases, where junctions formed between the lateral roots and the primary root (Figure 5, Panel B). In many observations a patch of “root surface”, where a number of bacterial cells were detected, often turned out to be some root hairs when observed from another angle. Often

can be seen that the bacteria grew along (Figure 5, Panel C) or even circling root hairs (Figure 5, Panel D). Therefore, root hairs appeared to be a most popular habitat for FZB42 growing on this segment.

Scanning electron microscopy (SEM) confirmed the presence of FZB42 on root hairs, where the bacterial cells were usually associated with a wealth of presumed root exudates (Figure 6, Panel C). The rich nutrients provided by the exudates may account for the high occurrence of FZB42 on root hairs. Another impressive phenomenon shown by SEM was that most of FZB42 cells captured on primary roots located themselves in some concave parts of root surfaces (Figure 6, Panel A, B).

So far neither cross sections observed with CLSM nor those observed with transmission electron microscopy (TEM) (Figure 6, Panel D) proved existence of FZB42 cells in the epidermis layer of maize root, suggesting that FZB42 should mainly, at least on maize, be an epiphytic rhizobacterium.

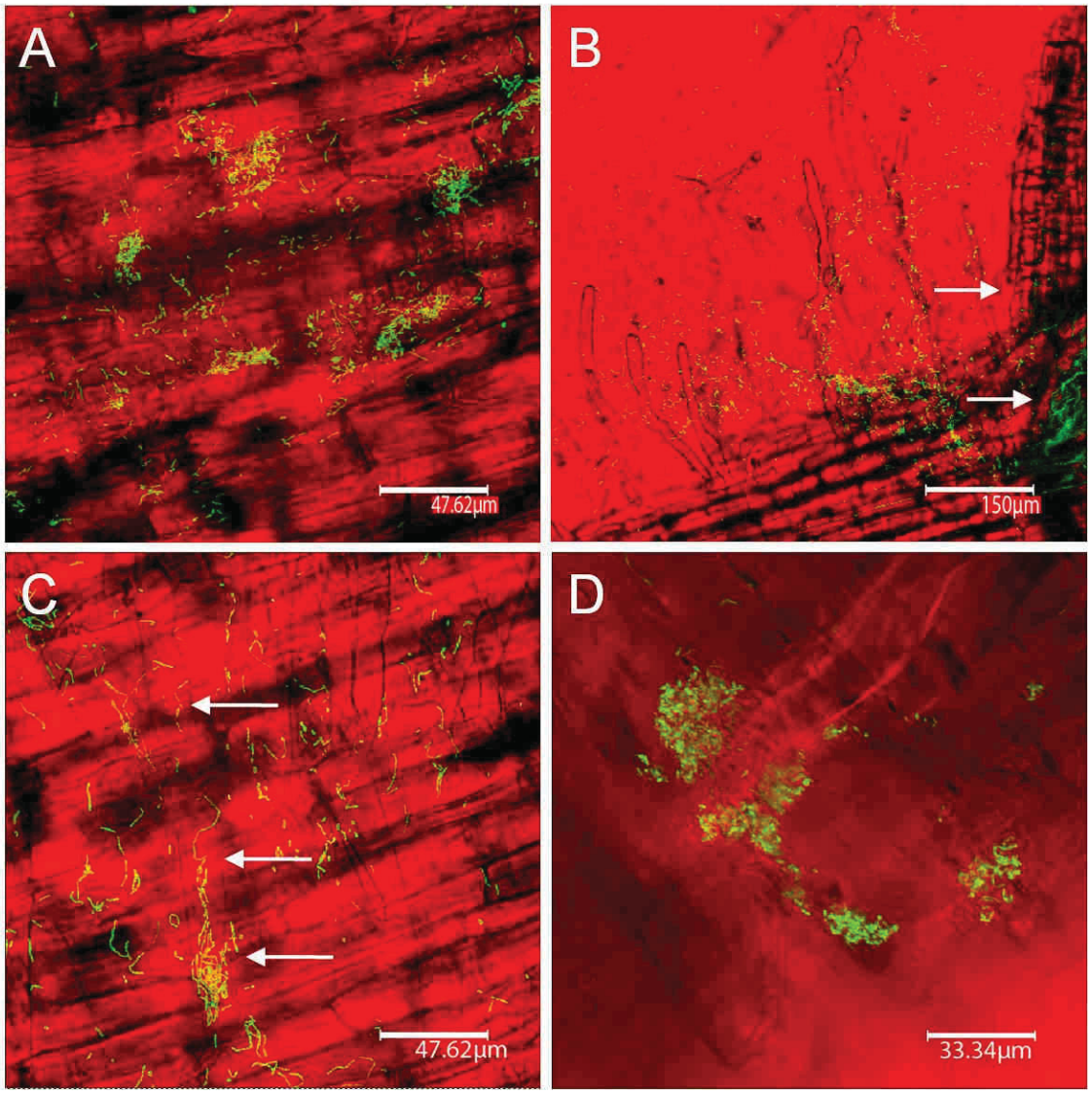


Figure 5: CLSM micrographs of GFP-labeled FZB42 colonizing maize roots in a gnotobiotic system. Panel A showed a larger view of FZB42 cells on the surface of a maize root. Note that the "surface" here may actually be some root hairs. As shown by arrows in Panel B, a heavily populated area by FZB42 was the junctions formed between primary roots and lateral roots. Panel C and D showed the bacteria closely associated with root hairs. Note that the bacterial cells growing along a root hair as indicated by arrows in Panel C.

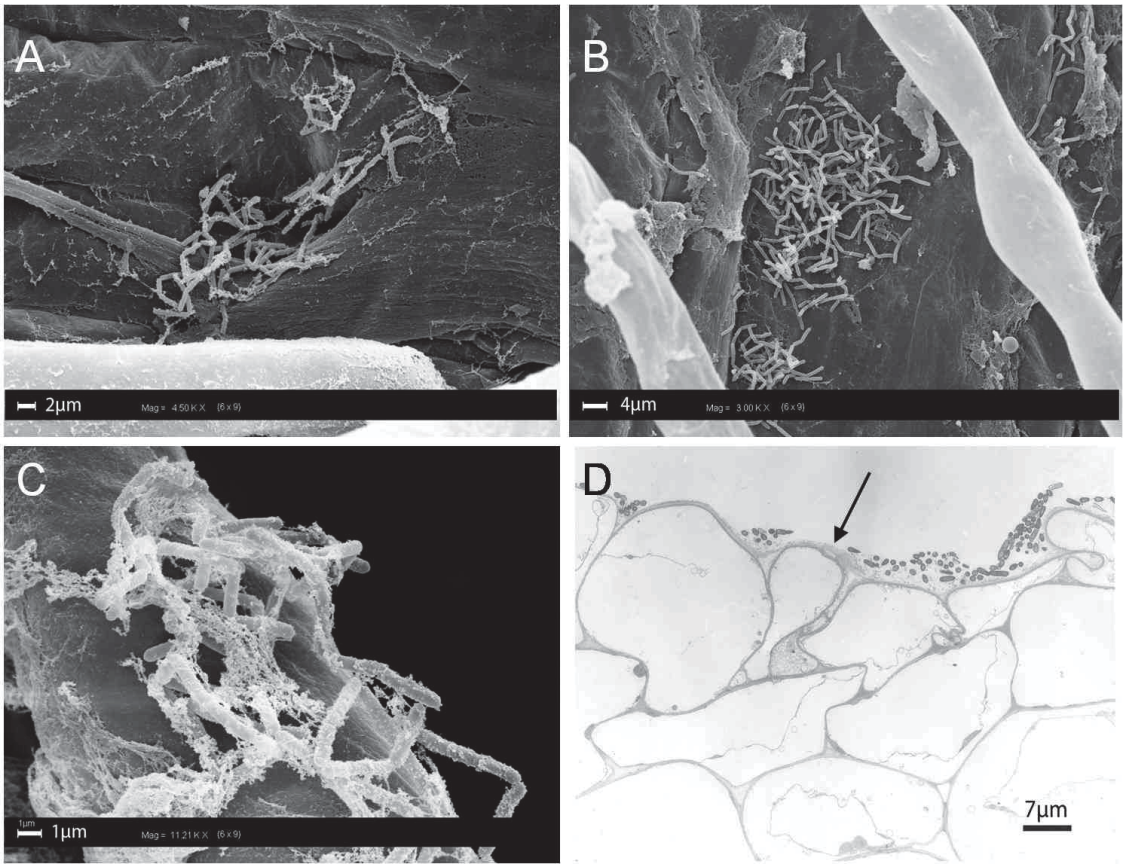


Figure 6: SEM (Panel A-E) and TEM (Panel F) micrographs of *B. amyloliquefaciens* FZB42 colonizing maize roots in a gnotobiotic system. Panel A and B recorded the presence of FZB42 cells on the concavities of root surfaces. Panel C showed a microcolony on the root hair. Note that the presumed root exudates associated with FZB42 cells in Panel C. TEM image (Panel F) revealed the FZB42 cells living outside the surface of a primary root. The arrows indicate the mucigel layer on the brim of the cross section.

3.1.5 Colonization of *Arabidopsis* by FZB42

After inoculation roots of *Arabidopsis thaliana* grew along the agar surfaces of MS basal medium. The primary roots reached around 5~6 cm in a week from the original length of 0.5~1.0 cm. The roots could easily be detached off from agar surfaces. After rinsing they were directly observed with microscope without making a section as was done with maize. The result showed that, like maize roots, root hairs of *Arabidopsis* were also significantly colonized by FZB42 (Figure 7, Panel A&B). On the other hand, unlike maize roots, primary root tips and lateral roots were other venues of *Arabidopsis* preferred by FZB42 (Figure 7, Panel C&D).

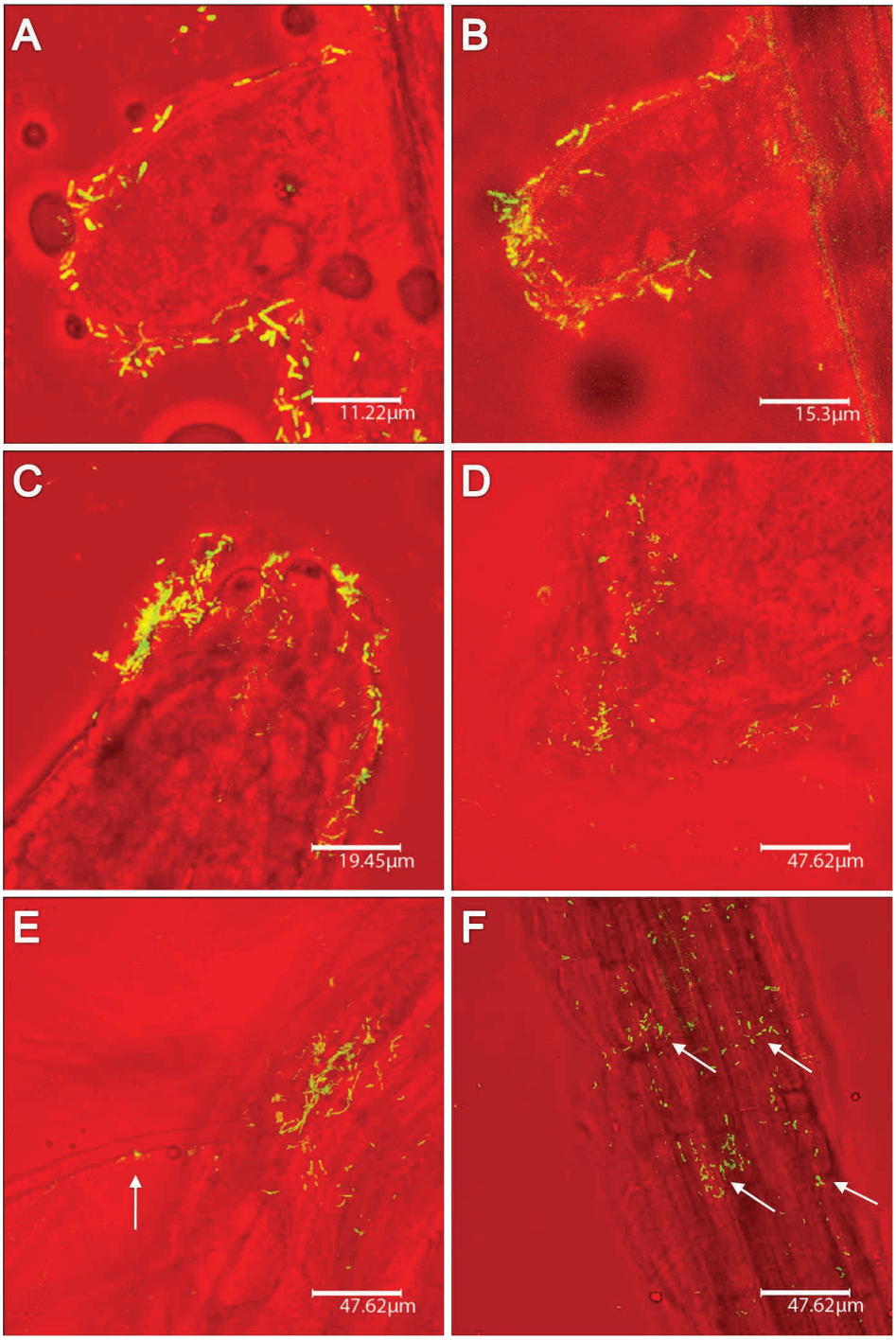


Figure 7: CLSM micrographs of GFP-labeled FZB42 colonizing *Arabidopsis* roots in a gnotobiotic system. Panel A&B showed the FZB42 associated with the emerging young roots hairs. Panel C&D showed bacterial cells colonizing the root tips. Panel E &F showed FZB42 cells colonizing *Arabidopsis* root surfaces. Note that the presence of FZB42 cells on a root hair as indicted by the arrow in Panel E and in the intercellular spaces between epidermis cells as indicted by the arrows in Panel F.

Interestingly, it was often recorded that FZB42 cells seemed to adapt themselves to the surface shape of root hairs (Figure 7, Panel A&B). This orientation should lead to an

intimate contact between bacterial cells and root hair surfaces so that the bacteria could, to maximum extent, immerse their bodies in the exudates secreted by root hairs. Another scenario which was often observed is that on root surfaces a significant portion of bacteria grew along or inside the boundary regions between epidermis cells as indicated by arrows in Panel F of Figure 7.

3.1.6 Colonization of *Lemna minor* by FZB42

Lemna minor ST is a species of *Lemna*ceae (duckweed family), which occurs broadly in natural environment of still waters from temperate to tropical zones. *L. minor* structurally consists of one, two or three fronds, each with a single root hanging in the water. It reproduces primarily by vegetative budding, occasionally by flowering [Armstrong 2010]. Unlike the roots of most other kinds of plants, *Lemna* roots contain rich chlorophyll while have no root hair [Cross 2002]. Due to its rapid propagation rate, *Lemna* has widely been used as an assay plant for many environmental investigations [Lyle Lockhart *et al*. 1989]. Here it is reported that FZB42 is able to colonize on *Lemna* and form robust biofilms.

One day after inoculation *L. minor* was rinsed twice and then viewed by CLSM. Fluorescent FZB42 cells could sporadically be found on *Lemna* fronds and roots, while a relatively high occurrence of colonization was observed on root tips (Figure 8, Panel A) and in connecting regions (Figure 8, Panel B) between roots and fronds. The preference of FZB42 to the two sites may be a suggestion that more nutrients or special compounds were present there, which were specifically recognized by FZB42 cells upon inoculation.

From the first day after inoculation, the Steinberg media were refreshed every other day as described in Materials and Methods. Five days after inoculation, a number of bacterial microcolonies could easily be observed on *Lemna* roots (Figure 8, Panel C) and fronds. In terms of the quantity of bacteria detected, the colonization of FZB42 on this day obviously appeared to be an intermediate phase between the situation of one day and that of nine days after inoculation as described above and below respectively.

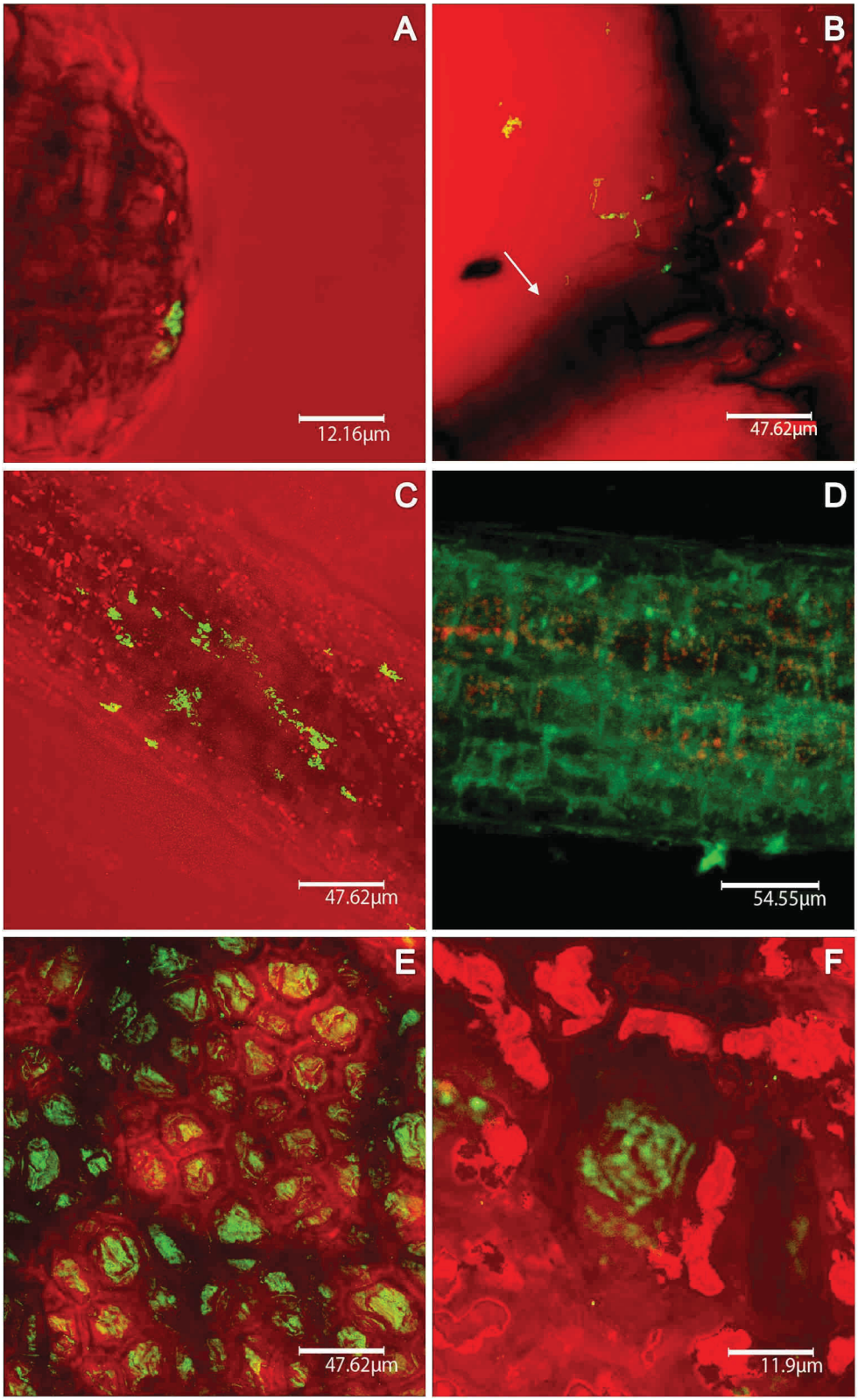


Figure 8: CLSM micrographs of *B. amyloliquefaciens* FZB42 colonizing *Lemna* minor ST. The colonization of *Lemna* by GFP-labeled FZB42 one day (Panel A&B), five days (Panel C), and nine days (Panel D-F) after inoculation were shown respectively. Note that one day after inoculation the FZB42 colonization mainly occurred on a root tip (Panel A) and in the intercellular spaces of linking regions between roots and fronds (Panel B, the root is indicated by the arrow). There were more microcolonies

on the roots of five days after inoculation (Panel C) than those of one day after inoculation. Robust biofilm could be found on some roots of nine days after inoculation (Panel D). In Panel E the large intercellular spaces are surrounded by layers of chloroplast-bearing parenchyma cells (in red) and almost each intercellular space in this area accommodated a FZB42 colony (in green). Panel F is a larger view of one intercellular space shown in Panel E.

Nine days after inoculation, FZB42 cells were found to colonize heavily some areas of roots or fronds of old *Lemna* plantlets whereas arise sporadically on those newly-emerged plantlets. In some areas of high colonization on ventral surfaces of *Lemna* fronds, the green fluorescent FZB42 cells formed colonies inside nearly each intercellular spaces surrounded by layers of chloroplast-bearing parenchyma cells (Figure 8, Panel E&F). On some segments of old roots the bacteria could even form a robust layer of biofilm (Figure 8, Panel D), the thickness of which was approximately 2 μm according to the analysis with software LCS 2.6.

SEM was also used to study the situation of nine days after inoculation. The result confirmed the observation with CLSM that most FZB42 cells on the ventral surfaces of *Lemna* fronds populated in the intercellular concaves formed by sack-like parenchyma cells (Figure 9, Panel A, B, and C). On some *Lemna* roots, it was clearly demonstrated that FZB42 cells grew along the grooves between epidermis cells (Figure 9, pane E &F). There was richer fluffy material in the grooves than elsewhere (Figure 9, Panel F). This kind of material, probably root exudates, was closely mixed with many bacterial clustered in the grooves.

The SEM micrographs have also displayed sophisticated biofilms developed on *Lemna*. In the biofilms many FZB42 cells altered their shapes from a smooth rod to a dumpy barrel, the diameter of which were approximately twice than that of the former shape (Figure 9, pane D, G &H). Meanwhile, the barrel-shaped cells were coated with a rough crust full of swellings and fiber-like structures (Figure 9, pane D, G &H). While the shorter fibers apparently served to link the nearby bacteria together (Figure 9, Panel D), the longer ones formed massively weaving the bacterial cells into a complex network (Figure 9, Panel G) or connecting them with *Lemna* surfaces (Figure 9, Panel H).

In the *Lemna* colonization studies, GFP-tagged *B. subtilis* 168 was also included as a reference strain. Unlike FZB42, nearly no colony of *B. subtilis* 168 could be detected on *Lemna* treated with the same preparation steps, corroborating the earlier reports about the

poor capability of domesticated *B. subtilis* to form robust biofilms [Branda *et al*. 2001; Kinsinger *et al*. 2003].

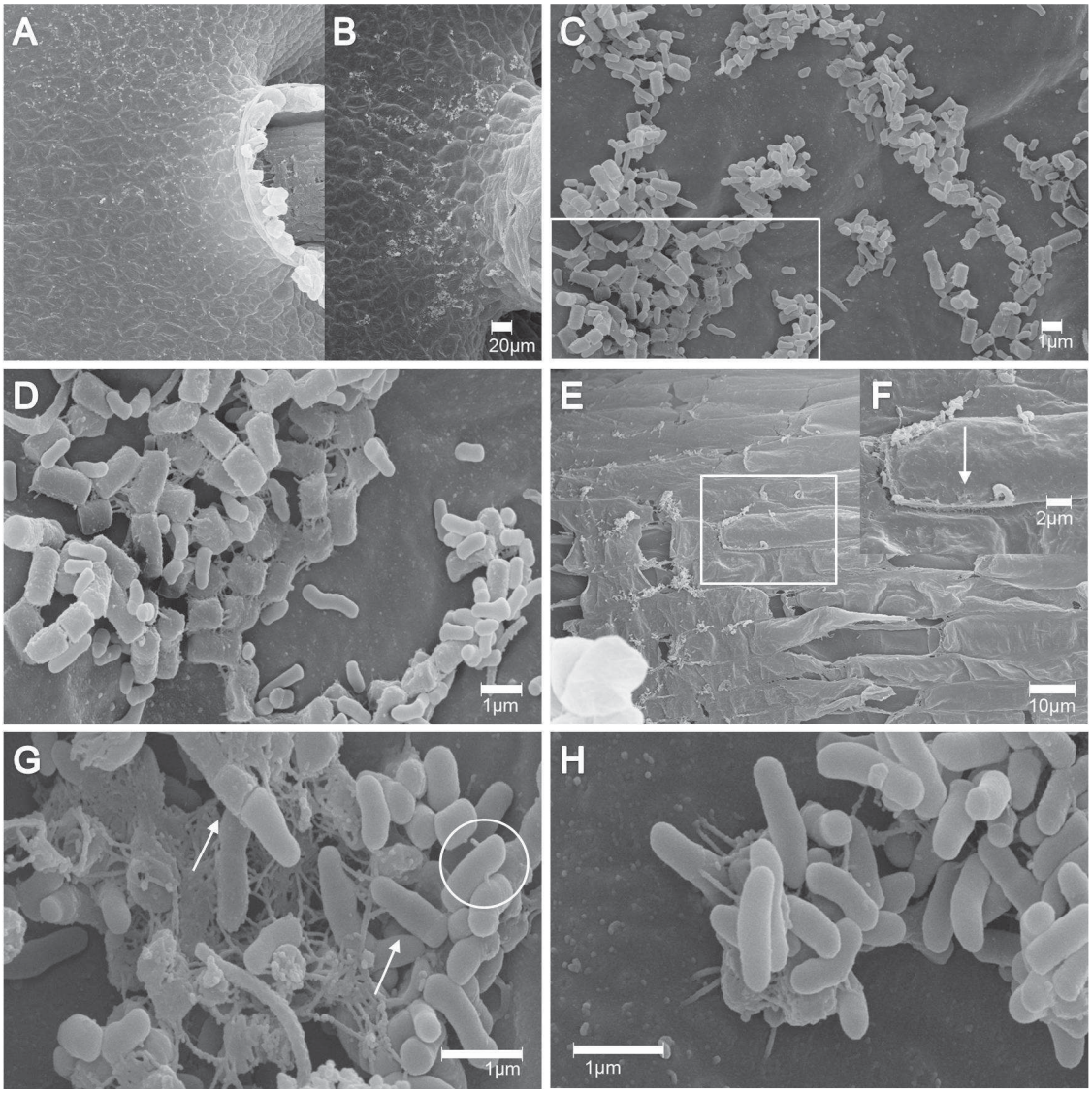


Figure 9: SEM micrographs of *B. amyloliquefaciens* FZB42 colonizing *L. minor* 9 days after inoculation in Steinberg medium. Panel A&B showed the colonization of FZB42 on the ventral surface of *Lemna* fronds near the frond-root linking region. Note that most FZB42 cells populated along the intercellular spaces between parenchyma cells. Panel C is an indented intercellular space surrounded by four parenchyma cells, while Panel D is an amplified view of the area enclosed by the rectangle in Pane C. Note the altered shape of many FZB42 cells and their rough coating structures shown in Panel D. Panel E&F showed the colonization of FZB42 on *Lemna* roots. Panel F is an amplified view of the area enclosed by the rectangle in Pane E. Note the bacterial cells populating along the grooves between the epidermis cells and the root exudates indicated by the arrow in Panel F. Panel G&H showed some details of the biofilms formed by FZB42 on *Lemna* fronds. Note the altering shape of FZB42 cells indicated by the arrows in Panel G and the fiber structures linking the bacteria together (in Panel G) or with the frond surface (Panel H).

3.2 Transcriptomic analysis of *B. amyloliquefaciens* FZB42 in response to maize root exudates

3.2.1 Assay of the compositions of maize root exudates

The maize root exudates used in this work were assayed with HPLC for the compounds such as organic acids, amino acids, and sugars, which are previously reported to be the major ingredients of root exudates [Simons *et al*. 1996; Lugtenberg *et al*. 1999; Lugtenberg *et al*. 2001; Rudrappa *et al*. 2008]. Among the three groups assayed (Figure 10) several organic acids such as lactic acid, malic acid, malonic acid, succinic acid and trans-aconitic acid, were the most abundant components in the exudates. There were also a variety of amino acids, which were less varying in amount but also less abundant than the organic acids. Among the sugars present in the exudates, glucose, melibiose, maltose, isomaltose, and lactose were relatively rich, especially the first two ones. Additional sugars were tested for their occurrence (xylose, palatinose, galactose, ribose and erythritol); however, these sugars were even less than arabinose in amount and thus not included in Figure 10. The assay was performed by Dr. Dmitriy Fedoseyenko at Institute of Plant Nutrition, University of Hohenheim.

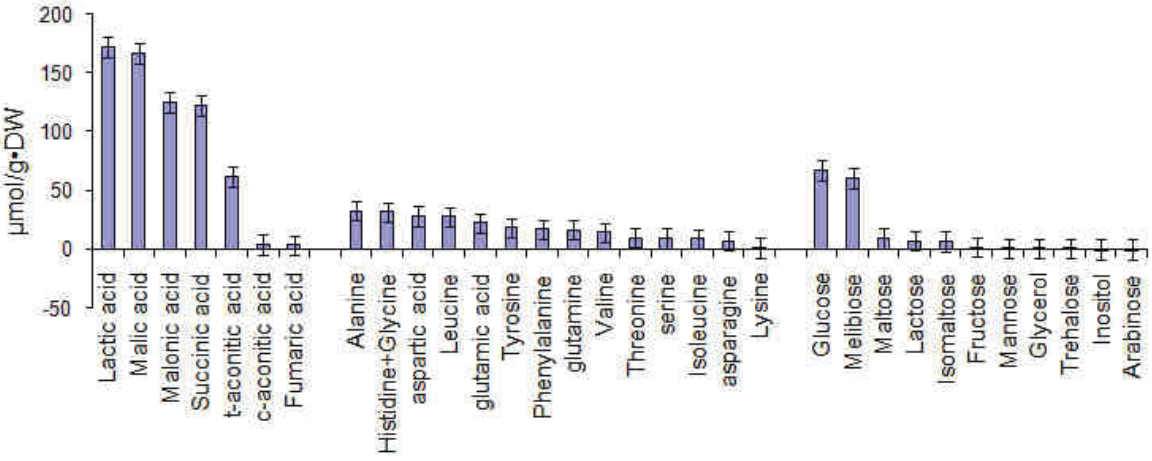


Figure 10: The compositions detected in the maize root exudates. The exudates were collected from the third day until the eighth day after maize seedlings were transferred to tubes. Three groups of components (organic acids, amino acids, and oligosaccharides) were tested by HPLC for their amounts. Relatively, several organic acids were the most abundant components detected. There were various amino acids, most of which are in a similar amount. Glucose and melibiose were the two most abundant oligosaccharides.

3.2.2 Experimental designs and transcriptomic data preprocessing

Besides the wild type strain, FZB42-derived mutants defective in the genes encoding alternative sigma factor such as SigB, SigD, SigM, SigV and SigX, and global transcriptional regulator DegU and AbrB were also analyzed by microarray in a similar manner. All experimental designs used in this work were shown in Table 7.

The microarray designed for *B. amyloliquefaciens* FZB42 in this work was designated Bam4kOLI (see Materials and Methods 2.5.4). Except for 28 various control probes, the array contains 3693 55mer oligonucleotides for probing the known or predicted protein-encoding genes of FZB42 and 238 70mer oligonucleotides for detecting the intergenic regions where putative small non-coding RNAs were encoded. The oligonucleotide probes were designed by Dr. Anke Becker at CeBiTec, Bielefeld University.

The transcriptomic data obtained were preprocessed in the procedures as followed. The genes with a q-value of ≤0.01 were firstly selected out, which were significantly differentially expressed according to statistics (see 2.5.7). The second cutoff, fold change (FCH) greater than 2.0, i.e. $M \geq 1.0$ or ≤ -1.0, was applied to most analyses. Only those meeting both filter conditions were regarded to be significantly differentially expressed and were chosen for further analysis. In the cases where more than three biological replicates were comprehensively analyzed, the threshold of FCH was set to be lower than 2.0, as specified in later sections.

Table 7: All pairs of transcriptomic profiling comparison designed in this work

Experiment	vs.	Control	Nr. of biological replicates	FCH applied
wt+RE_1.0	<>	wt-RE_1.0	3	≥2
wt+RE_3.0	<>	wt-RE_3.0	6	≥1.5
degU+RE_3.0	<>	degU-RE_3.0	3	≥2
abrB+RE_3.0	<>	abrB-RE_3.0	3	≥2
sigB+RE_3.0	<>	sigB-RE_3.0	3	≥2
sigD+RE_3.0	<>	sigD-RE_3.0	3	≥2
sigM+RE_3.0	<>	sigM-RE_3.0	3	≥2
sigV+RE_3.0	<>	sigV-RE_3.0	3	≥2
sigX+RE_3.0	<>	sigX-RE_3.0	3	≥2
degU+RE_3.0	<>	wt+RE_3.0	3	≥1.5‡

abrB+RE_3.0	<>	wt+RE_3.0	3	≥2
sigB+RE_3.0	<>	wt+RE_3.0	3	≥2
sigD+RE_3.0	<>	wt+RE_3.0	3	≥1.5‡
sigM+RE_3.0	<>	wt+RE_3.0	3	≥2
sigV+RE_3.0	<>	wt+RE_3.0	3	≥2
sigX+RE_3.0	<>	wt+RE_3.0	3	≥2
wt+SE_1.0	<>	wt-SE_1.0	3	≥2*
wt+SE_3.0	<>	wt-SE_3.0	3	≥2*
degU+SE_3.0	<>	wt+SE_3.0	3	≥1.5*‡
sigD+SE_3.0	<>	wt+SE_3.0	6	≥1.5*‡
wt+IE_3.0	<>	wt+RE_3.0	3	≥2

Remarks: Abbreviations used in Table 7 represents, respectively: wt: FZB42 wild type; degU: FZB42 Δ*degU*; abrB: FZB42 Δabr*B*; sigB: FZB42 Δ*sigB*; sigD: FZB42 Δ*sigD*; sigM: FZB42 Δ*sigM*; sigV: FZB42 Δ*sigV*; sigX: FZB42 Δ*sigX*; RE: root exudates; SE: soil extract; +: in the presence of root exudates or soil extract; -: without root exudates or soil extract; 1.0: cells were collected when OD_{600}=1.0; 3.0: cells were collected when OD_{600}=3.0; IE: "interaction exudates"; *: the basal media used were 1C media instead of 1CS media; ‡: special processing procedures were applied, refer to section 3.3.2 and 3.3.5.

3.2.3 Determination of the microarray experimental conditions

The first step of microarray experiments was to determine an appropriate concentration at which the exudates should be applied and to determine proper time points when the bacterial cells should be harvested. The criterion of this determination is that the two conditions, when applied, should result in a significant effect on FZB42 and therefore its transcriptional response to the exudates can easily be detected by means of microarray. Based on the previous proteomic work of FZB42 [Chen *et al*. 2007], three concentrations (0.25 mg/ml, 0.5 mg/ml and 1.0 mg/ml) of the exudates and two time points (OD_{600}=1.0 and OD_{600}=3.0, for the reason of simplicity, throughout this work the two time points were referred as OD1.0 and OD3.0 respectively) were tested in a pilot experiment. With the cutoff of q≤0.01, only a few genes of the cells harvested at the early exponential phase (OD1.0) were altered in transcription, while hundreds of genes were significantly altered during the late exponential phase (Figure 11). At OD3.0, the number of genes up-regulated

by the exudates decreased gradually along with the increase of exudates concentration, suggesting that high concentration of exudates may repress the expression of some genes of FZB42. As a consequence, the concentration of 0.25 mg/l and the cell density of OD3.0 were used for most of the later microarray experiments.

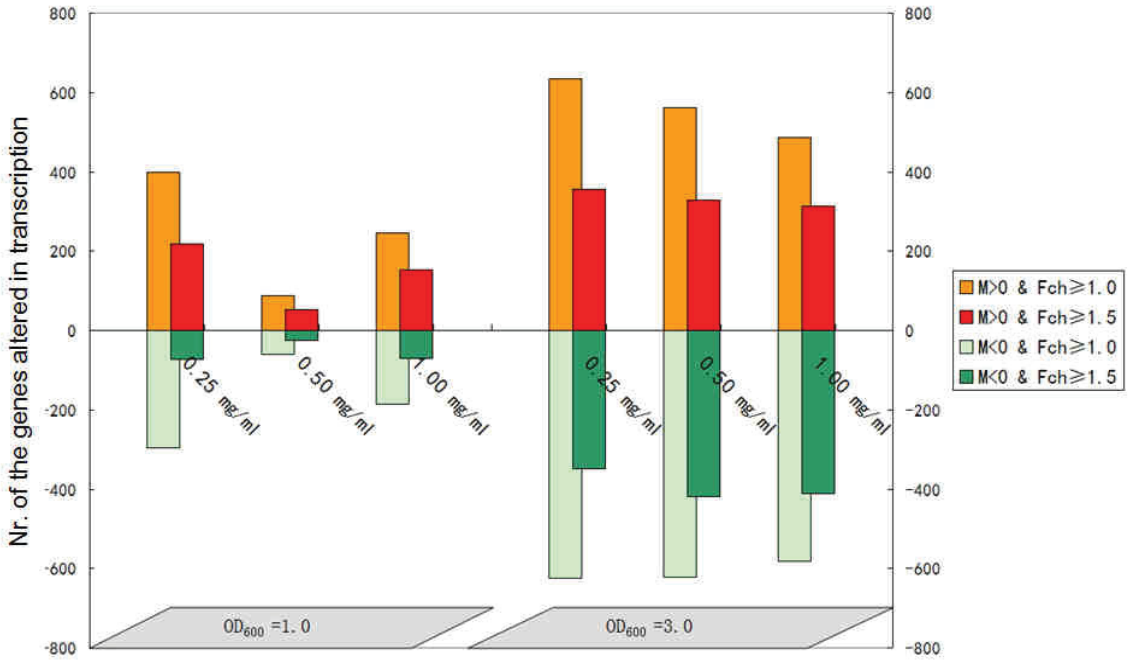


Figure 11: Number of genes altered in transcription in response to root exudates under different conditions.

3.2.4 A general profiling of the genes which were altered in expression by root exudates

The most important task of this work was to identify the genes of FZB42 involved in plant-microbe interaction. No gene was affected at early exponential phase (OD1.0) by the presence of root exudates, when the conditions were set to be q≤0.01 and FCH≥2.0. At the transient phase (OD3.0), six biological replicates were analyzed comprehensively (q≤0.01 and FCH≥1.5). The result showed that a total of 302 genes (Appendix Table 1, Appendix Table 2, and Appendix Table 3), representing 8.2% of the transcriptome, were significantly regulated by root exudates. The majority of these genes (260) were up-regulated, whereas only 42 genes were down-regulated (Figure 12). Although most of the regulated genes

have been annotated with a known function, a significant proportion (~23%) of the genes remains unknown in function so far, among which 19 genes are unique to FZB42. In addition, 44 genes (~15%) encode either hypothetical proteins or proteins with putative functions (Figure 12).

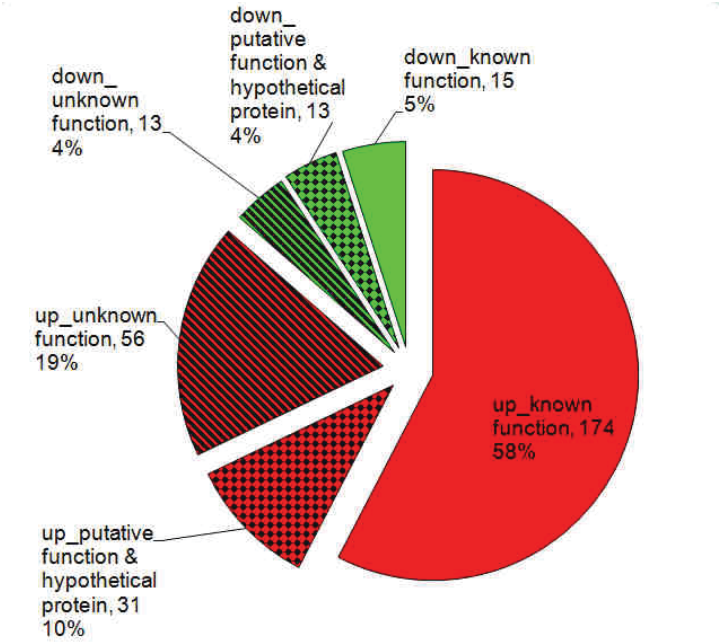


Figure 12: An overview of various groups of genes altered in their expression by root exudates. "Up" means the genes which were up-regulated. "Down" means the genes which were down-regulated.

3.2.5 Validation of the microarray data by real time PCR

Ten out of the 302 genes were chosen, covering the different levels of fold change according to the transcriptomic result, to be evaluated by real-time PCR for their response to root exudates. Except for one gene (*bcd*), all others were validated by real-time PCR to have a significant alteration in expression (Figure 13). Furthermore, most of the genes showed a more or less similar fold changes to that obtained in microarray experiments (Appendix Table 1).

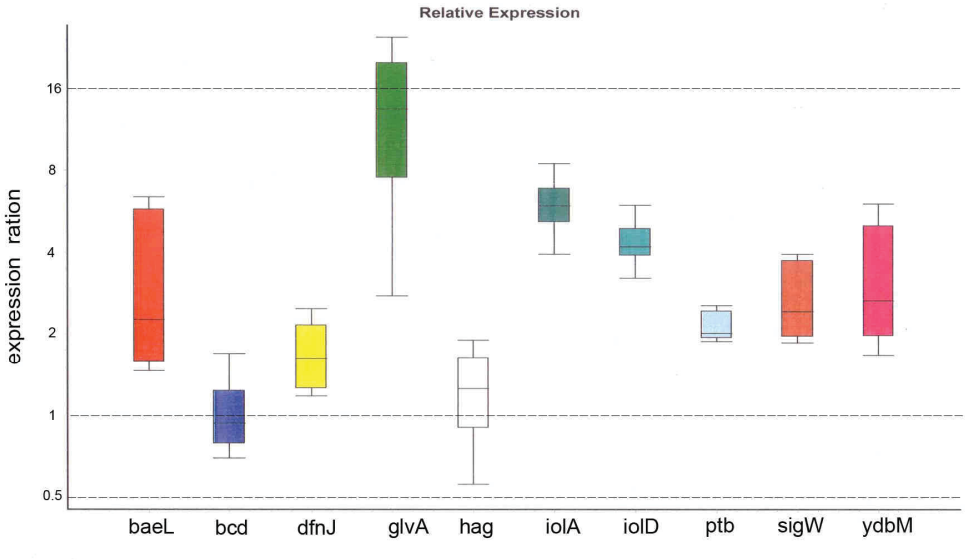


Figure 13: Expression ratio of selected genes in the presence of root exudates to those in the absence of root exudates.

When the microarray data were compared with the results of proteomic studies performed by Kinga Kierul in our laboratory, 18 genes were found to be regulated by root exudates at both transcriptomic and proteomic level (Table 8). While only the gene *alaS* was down-regulated in both data, 17 of them were up-regulated. Among the 17 genes, 15 encoded cytosolic proteins and two encoded proteins of the secretome.

Table 8: The genes regulated by root exudates in both transcriptomic and proteomic results

Gen	***Product***	***FCH***	
		in Transcriptome	***in proteome***
citB	aconitate hydratase CitB	1.7	2.1
rocF	arginase RocF	5.4	3.0
pgi	glucose-6-phosphate isomerase	1.5	1.8
lepA	GTP-binding protein LepA	1.5	3.0
grp	heat-shock protein GrpE	1.5	1.5
infB	initiation factor (IF-2) InfB	1.6	1.7
iolB	inositol utilization protein B (IolB)	2.7	1.6
iolI	inositol utilization protein I (IolI)	2.0	2.2
ype	sporulation protein YpeB	1.5	1.8
fusA	elongation factor G FusA	2.2	1.1
tufA	elongation factor Tu TufA	1.5	1.9
bcd	leucine dehydrogenase Bcd	1.8	1.7
mdh	malate dehydrogenase Mdh	1.9	1.5
glvA	maltose-6'-phosphate glucosid	5.2	2.4

pgk	phosphoglycerate kinase Pgk	2.4	1.6
alaS	alanyl-tRNA synthetase AlaS	-1.5	0.6
pdh	pyruvate dehydrogenase PdhC	1.5	2.0
opp	oligopeptide ABC transporter	1.5	2.3

3.2.6 The regulated genes with known function

Among the 302 genes, which were significantly altered in transcription by root exudates, 189 were annotated with known function. They were categorized in various classes [Moszer et al. 2002] such as cell envelope and cellular processes, intermediary metabolism, information pathway and other functions (Table 9, Appendix Table 1). Among these categories four groups, as highlighted in Table 9, were particular, because they contained a higher number of genes and more than one third of these genes in each group had a fold change of ≥2.0. The groups were specified as followed.

Table 9: The categories of genes regulated by root exudates with known function

fictional category	number
1_cell envelope and cellular processes	**58**
1.7_ Cell division	6
1.1_ Cell wall	5
1.4_ Membrane bioenergetics	7
1.5_ Mobility and chemotaxis	6
1.3_ Sensors (signal transduction)	2
1.6_ Protein secretion	5
1.8_ Sporulation	7
1.1_ Transformation/competence	2
1.2_ Transport/binding proteins and lipoproteins	18
2_intermediary metabolism	**59**
2.1_ Metabolism of carbohydrates and related molecules	34
2.2_ Metabolism of amino acids and related molecules	12
2.5_ Metabolism of coenzymes and prosthetic groups	4
2.4_ Metabolism of lipids	5
2.3_ Metabolism of nucleotides and nucleic acids	4
3_information pathways	**45**
3.3_ DNA recombination	1
3.1_ DNA replication	3
3.8_ Protein modification	2
3.7_ Protein synthesis	20
3.6_ RNA modification	1

3.5_ RNA synthesis	18
4_other functions	**27**
4.1_ Adaptation to atypical conditions	6
4.2_ Detoxification	4
4.6_ Miscellaneous	3
4.4_ Phage-related functions	1
4.3_ Antibiotic production	13

3.2.6.1 The genes involved in nutrition utilization

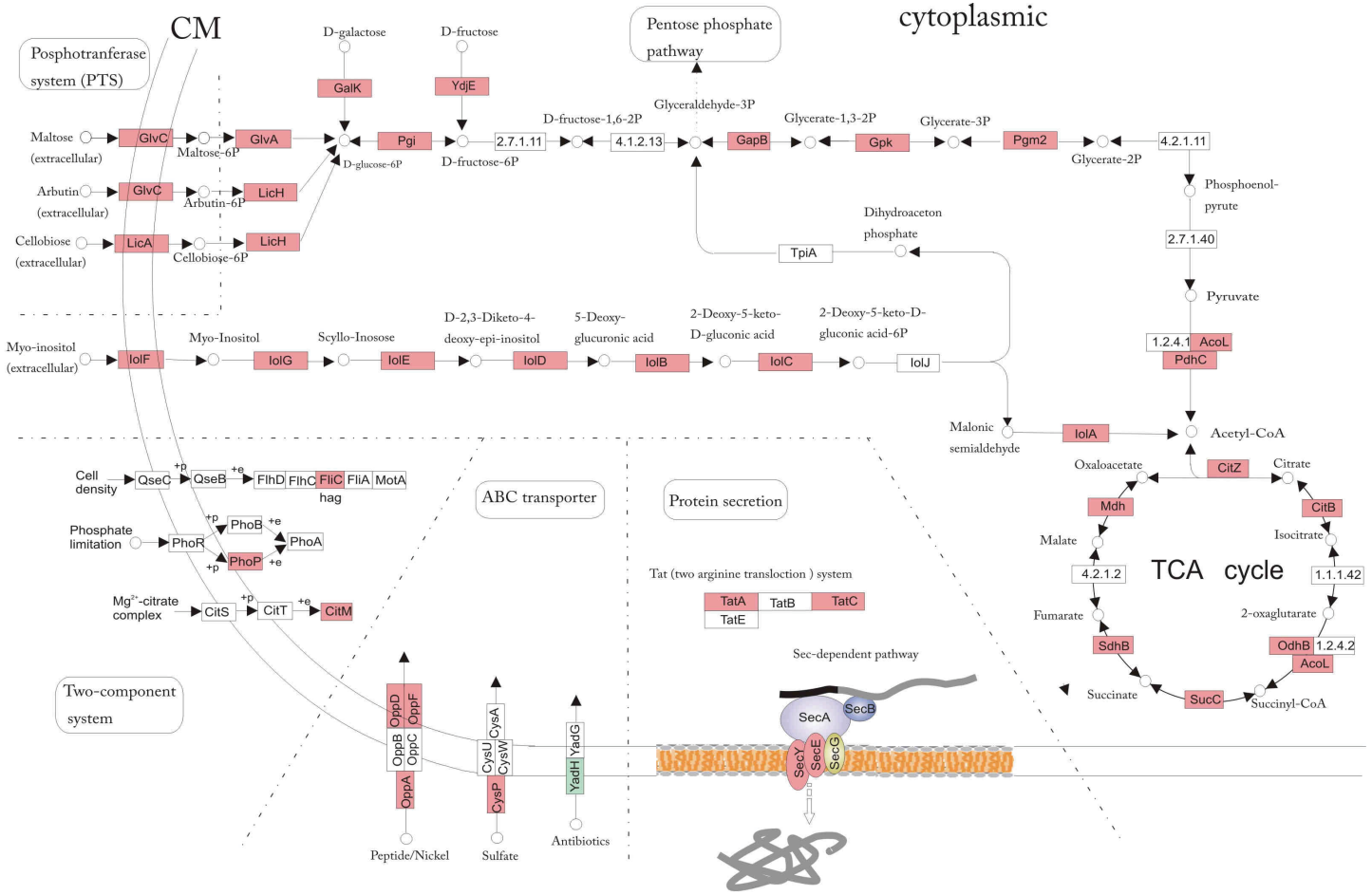
The transcriptions of 46 genes, 43 being up-regulated, were changed in response to the root exudates. The genes were involved in different aspects of metabolism of carbohydrates, amino acids and related molecules. In order to have a deeper understanding of relationships among them, the genes were mapped in the KEGG pathway. A diagram was accordingly constructed (Figure 14). A total of 12 genes encoding enzymes involved in EMP pathway (counting from *pgi* encoding for glucose-6-phosphate isomerase) and TCA cycle were significantly up-regulated. These genes cover almost the entire circuit for glycolysis and energy generation. Furthermore, if taking into account of another 11 of 18 genes encoding transport/binding proteins and lipoproteins (Table 9), approximately 30% of the genes with known function contributed to uptake or utilization of nutrient molecules.

This finding is perhaps not surprising since monosaccharides, amino acids, and organic acids are thought to be the major constituents of plant root exudates [Simons *et al*. 1996; Lugtenberg *et al*. 1999; Lugtenberg *et al*. 2001]. Utilization of organic acids was shown to be the nutritional basis for the ability of *Pseudomonas fluorescens* to colonize tomato roots. Some genes in *Pseudomonas* encoding proteins with function in nutrient assimilation and in energy production are reported to be up-regulated in the rhizosphere or when bacteria were exposed to the soil environment as demonstrated by *in vivo* expression technology-based approaches [Silby *et al*. 2004; Ramos-Gonzalez *et al*. 2005]. Here a significant portion of the up-regulated genes in FZB42 were also found to be devoted to nutrient utilization and energy generation.

Among the up-regulated genes three of them, *glvA, glvC and glvR*, were the ones with the highest fold change (glvA: 5.2-fold ↑, glvC: 2.5-fold ↑, glvR: 4.4-fold ↑). The enhancement of *glvA* expression was also validated by real-time PCR as well as by proteomic approach (Kinga Kierul). These three genes compose of *glv* operon (*glvA-glvR-*

glvC) and are positively regulated by maltose [Yamamoto *et al*. 2001]. The significant up-regulation of these genes suggested that maltose should be present in the exudates, which has been demonstrated by HPLC profiling (Figure 10).

The genes involved in inositol metabolism (*iolA, iolB, iolC, iolD, iolE, iolF, iolG, iolI, iolS*) were also up-regulated, mainly with a fold change of ≥2.0 (Figure 14 and Appendix Table 1). Except *iolS,* which may be involved in regulation of inositol catabolism, the other eight genes are all members of *iol* operon. The increased transcription of *iolA* and *iolD* was confirmed by real-time PCR while the enhancement of *iolB* and *iolL* was validated by proteomic profiling (Kinga Kierul). The activation of the nine genes indicated the presence of inositol in the exudates, which has also been detected by HPLC, although in a relatively low amount.



oppA,oppF: hyperthetical protein

yadH: putative ABC transporter

Figure 14: The functions of a subset of up-regulated gene with known function in response to maize root exudates.

3.2.6.2 The genes involved in chemotaxis, motility and biofilm formation

Besides those involved in nutrient utilization, a second group of genes with a higher fold change are associated with sensors, chemotaxis, motility and biofilm formation (Table 10). These processes are crucial for bacterial colonization on plants.

Table 10: The induced genes involved in mobility and chemotaxis

Gene	*Fold change*	*Classification code_function involved*
fliM	2.0	1.5_ Mobility and chemotaxis
fliP	1.7	1.5_ Mobility and chemotaxis
cheC	1.7	1.5_ Mobility and chemotaxis
cheD	-1.5	1.5_ Mobility and chemotaxis
hag	3.6	1.5_ Mobility and chemotaxis
flgM	1.7	1.5_ Mobility and chemotaxis
luxS	1.7	1.3_ Sensors (signal transduction)
ymcA	2.5	1.3_ Sensors (signal transduction)

In nature, recognizing signals emitted from each other by bacteria and by plants is the first step of their cross-talk [Bais *et al*. 2004]. For bacteria, once perceiving signals of a plant nearby, the mobilization towards to the plant establishes the basis for their further relationship [O'Sullivan *et al*. 1992; Walsh *et al*. 2001; de Weert *et al*. 2002; de Weert *et al*. 2004]. Bacterial movement from soil to plants or their spreading over root surfaces involves several factors such as chemotaxis, flagella-driven motility, swarming process, and production of surfactants [Daniels *et al*. 2004; Raaijmakers *et al*. 2006; Ongena *et al*. 2008]. Therefore, activation of the genes required for chemotaxis (*cheC, cheD*) and flagellar formation or motility (*hag, fliD, fliP* and *flgM*) provided an indirect evidence that biological processes of *Bacillus* involved in plant-microbe interactions are mediated by some components present in root exudates.

Forming biofilm on plant roots is a prerequisite of efficient colonization by PGPR. Biofilms not only strengthen the interaction between plants and PGPR but also provide plant root system with a protective barrier against attacks of pathogenic microbes [Ongena

et al. 2008]. Here the transcription of two genes (*ycmA* and *luxS*) involved biofilm formation were induced by root exudates.

Gene *luxS* was indentified in both Gram-negative and Gram-positive strains [Surette *et al*. 1999; Jones *et al*. 2003]. It is required for the synthesis of quorum-sensing signaling molecule autoinducer-2 (AI-2) [Huang *et al*. 2009]. It was shown that LuxS is involved in biofilm formation of not only pathogenic *Streptococcus* sp. [Heilmann *et al*. 1996; Gotz 2002; Huang *et al*. 2009] but probiotic *B. subtilis* natto strain [Lombardia *et al*. 2006]. Compared with *luxS*, the function of *ycmA* had remained elusive until it was indentified to be involved in biofilm formation [Branda *et al*. 2004]. More recently, it was proposed that *ycmA* functions by antagonizing the repression mediated by SinR, a master regulator of biofilm formation [Kearns *et al*. 2005]. In this study the transcription of *luxS* and *ycmA* was up-regulated by root exudates, indicating that the formation of biofilm of FZB42 was enhanced by some signals in root exudates.

3.2.6.3 The genes involved in antibiotic production

The third group of genes induced by root exudates was those involved in synthesis of antimicrobial compounds (Table 11). Producing antibiotics against deleterious microbes in rhizosphere is an established mechanism for the beneficial effect of *B. amyloliquefaciens* FZB42 on plants [Chen *et al*. 2009; Chen *et al*. 2009; Chen *et al*. 2009]. Here the induced genes are mainly devoted to the synthesis of two polyketide antibiotics, bacillaene and difficidin. This indicates that some components in the exudates stimulated the production of the two antibiotics, which have been demonstrated to be able to protect orchard trees from fire blight disease caused by *Erwinia amylovora* [Chen *et al*. 2009].

Another two induced genes *mlnH* and *fenE* participate in the biosynthesis of macrolactin and fengycin, respectively. Macrolactin is a third polyketide product found in FZB42 and has activity against some Gram-positive bacteria [Schneider *et al*. 2007], while fengycin was shown to act against phytopathogenic fungi in a synergistic manner [Koumoutsi *et al*. 2004; Chen *et al*. 2009].

Table 11: The root exudates-induced genes involved in antibiotic production

Gene	Product	FCH
baeE	malonyl-CoA-[acyl-carrier protein] transacylase	1.6
baeI	enoyl-CoA-hydratase BaeI	2.2
baeL	polyketide synthase BaeL	1.9

baeN	hybrid NRPS/PKS BaeN	1.5
baeR	polyketide synthase BaeR	2.3
dfnJ	modular polyketide synthase of type I DfnJ	2
dfnI	modular polyketide synthase of type I DfnI	1.7
dfnG	modular polyketide synthase of type I DfnG	2
dfnF	modular polyketide synthase of type I DfnF	2.4
mlnH	polyketide synthase of type I MlnH	1.5
fenE	fengycin synthetase FenE	1.5
srfAD	surfactin synthetase D SrfAD	1.9
srfAC	surfactin synthetase C SrfAC	1.7

Surfactin synthetase of *Bacillus* comprises four large open reading frames (ORFs) designated *srfAA*, *srfAB* , *srfAC* and *srfAD* respectively [Peypoux *et al*. 1999; Lee *et al*. 2007]. At least two genes for the synthetase were activated by root exudates (Table 11). Like fengycin, surfactin is one of *Bacillus* cyclic lipopeptides. It displays antiviral and antibacterial activities but, in contrast to fengycin, no significant fungitoxicity. The ability of surfactin to reduce the invasion of *Pseudomonas syringae* on *Arabidopsis* plants has been reported [Bais *et al*. 2004], however, it is not yet clear whether the protective effect is caused directly from its antibacterial activity or from its another biofilm-relating property.

Surfactin is crucially involved in the surface motility of *Bacillus* by reducing the surface tension [Daniels *et al*. 2004; Leclere *et al*. 2006; Raaijmakers *et al*. 2006] and contribute to the biofilm spreading on *Arabidopsis* roots [Bais *et al*. 2004]. As discussed previously, PGPR forming a robust biofilm can prevent the deleterious microbes from adhering to root surfaces or inhibit biofilm developing of pathogenic cells. The enhanced transcription of *srfAC* and *srfAD* by root exudates (Table 11) indicated induced surfactin production, which would, therefore, contribute to the protective role of FZB42 against plant pathogens.

Besides the groups described above, there were still many differentially expressed genes, some of which were involved in interesting functions or circuits. For instance, gene *scoB* and *yngG* are known to be involved in synthesis and degradation of ketone bodies (Figure 15). Gene *scoB* was up-regulated while yngG was down-regulated (Figure 15), suggesting that accumulation of acetoacetate (AcAcO) may occur in FZB42 cells. Ketones are an important class of volatile organic compounds (VOCs) related to plant-microbe interactions [Ryu et al. 2003; Steeghs et al. 2004; Zhang et al. 2007]. One of the ketones, acetoin, has been demonstrated to be able to trigger induced systemic resistance (ISR) of

Arabidopsis and promote its growth [Ryu et al. 2003; Ryu et al. 2004; Rudrappa et al.]. As small molecular ketone analogues, AcAcO itself or its derived metabolites such as acetone, butanal or butanol might also be involved in plant-microbe interactions. This postulation needs be tested in further studies.

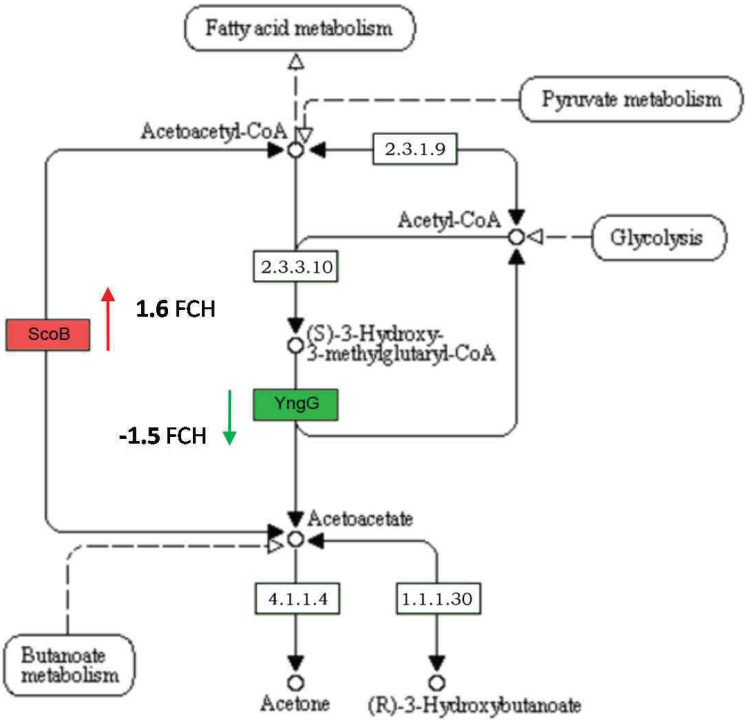


Figure 15: The genes involved in synthesis and degradation of ketone bodies. The transcription of *scoB* was up-regulated with a 1.6 FCH and therefore highlighted in red. The transcription of *yngG* was down-regulated with a 1.5 FCH and therefore highlighted in green.

3.2.7 The regulated genes with putative function

Out of the 302 genes altered significantly in transcription by root exudates, 44 encoded a putative enzyme or a hypothetical protein. Among them a few genes are noteworthy because their functions may be involved in plant-microbe interactions. Gene *ydjL*, which was suggested to be renamed as *bdhA* [Nicholson 2008], encodes for a putative dehydrogenase catalyzing a reversible reaction: Acetoin + NADH ↔ 2,3-butanediol + NAD^+. The 2, 3-butanediol is one kind of VOCs released by PGPR and demonstrated to be able to significantly promote plant growth [Ryu *et al*. 2003]. The expression of gene *epsE* residing in a 15-gene operon *epsA-O* is also enhanced by root exudates. EpsE is involved in formation of biofilm by arresting flagellar rotation of cells

embedded in the biofilm matrix [Blair *et al*. 2008]. Another activated gene *dfnY* is predicted to encode a hypothetical protein. Like other induced genes with known production such as *dfnF*, *dfnG*, *dfnI*, and *dfnJ* (Table 11), *dfnY* is one component of the gene cluster responsible for synthesis of the polyketide antibiotic difficidin. The three large categories into which the genes with putative function fall (Appendix Table 2) are metabolism of carbohydrates and related molecules, metabolism of amino acids and related molecules, and transport/binding proteins and lipoproteins. This is a similar result to the genes with known function (Table 9). Figure 16 summarizes the distribution of all genes, with known and putative function, in various functional categories.

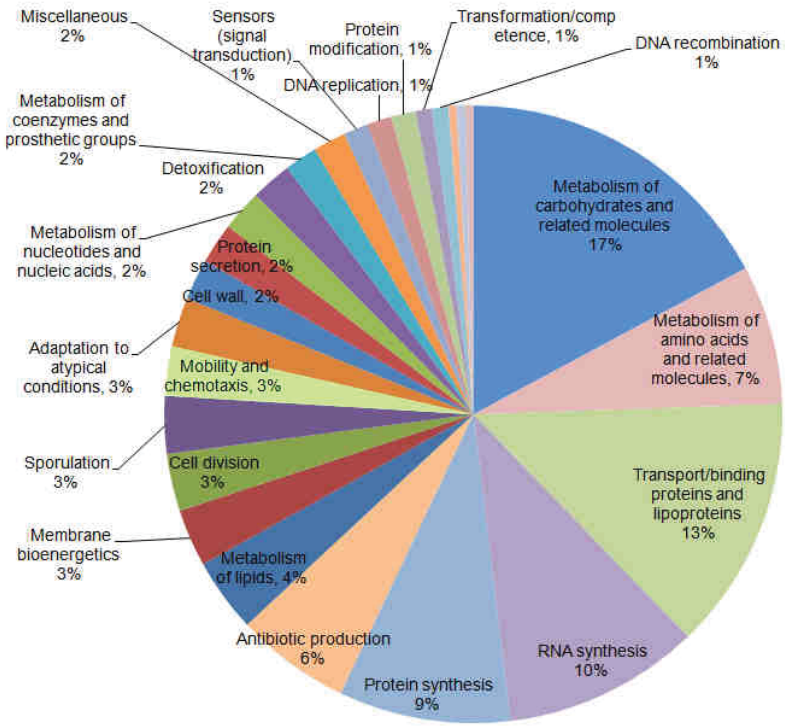


Figure 16: The distribution in various functional categories of all genes with known and putative products, which were altered in transcription by root exudates.

3.2.8 The effect of soil extract on FZB42 transcriptome

In order to provide an environment resembling to rhizosphere, soil extract (SE) was included in most media used in this work. The effect of soil extract on gene expression of FZB42 was likewise examined by microarray. The RNAs isolated from cells grown in 1C medium in the presence of soil extract, at OD1.0 and OD3.0 respectively, were compared with that absent of soil extract. The result showed that no gene was significantly regulated

by the soil extract at growth phase of OD1.0 while the expression of five genes was repressed by soil extract at OD3.0, as shown in Table 12.

Table 12: The repressed genes of FZB42 by soil extract at the growth phase when OD_{600}=3.0

Gene	FCH	Product	Function involved
ypeQ	-2.6	hypothetical protein YpeQ	unknown
yurV	-2.4	iron-sulfur cofactor synthesis protein nifU homolog YurV	miscellaneous
iolS	-2.2	inositol utilization protein S (IolS)	metabolism of carbohydrates and related molecules
yaaA	-2.0	conserved hypothetical protein YaaA	unknown
ahpF	-2.0	alkyl hydroperoxide reductase (large subunit) and NADH dehydrogenase AhpF	detoxification

3.2.9 Clustering analysis

Clustering is an analysis method often used for microarray data. The genes which are closely related in function are often shown to be regulated in a coordinated manner in response to environmental stimuli so that the genes would be "clustered" into one group in clustering analysis [Eisen *et al*. 1998; Boorsma *et al*. 2005; Horan *et al*. 2008]. Therefore, clustering the regulatory response of a bulk of genes to a series of environmental conditions allows us to predict the function of an uncharacterized gene, based on the functions of other genes which are clustered in the same group. Hierarchical clustering is one of classical clustering algorithms and is most often used. A hierarchical clustering of the 302 genes was performed with software package Genesis [Sturn *et al*. 2002]. The overview of the clustering results is shown in Figure 17.

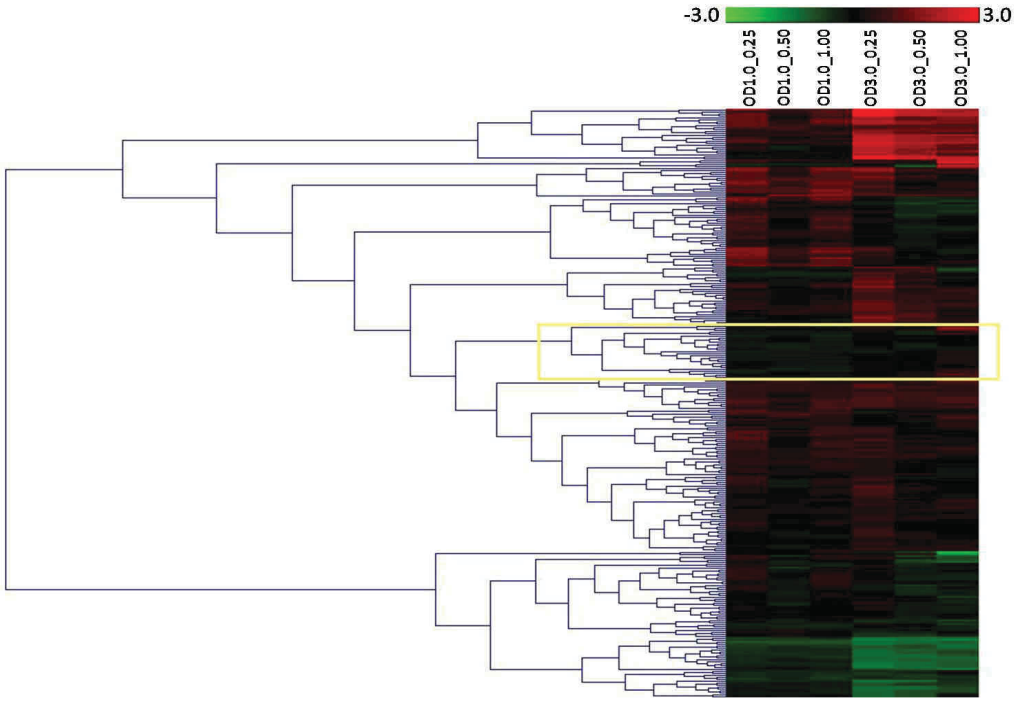
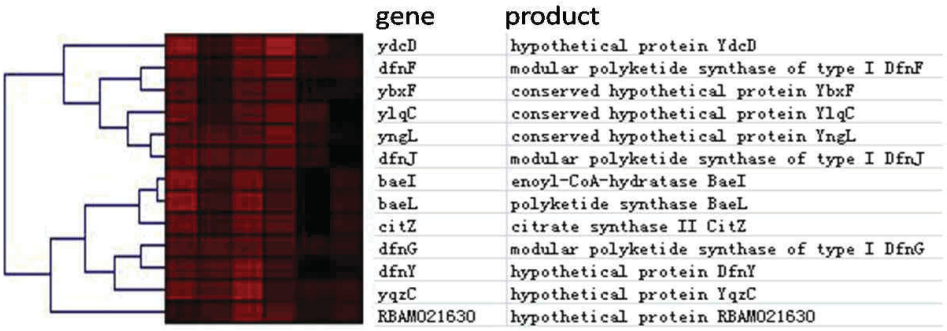


Figure 17: The heatmap of the hierarchical clustering result of the 302 genes which were significantly differentially expressed in response to root exudates. OD1.0_ 0.25 means that the cells were grown in 1CS medium added with 0.25 mg/ml root exudates and harvested when OD_{600}=1.0; similarly, OD3.0 means OD_{600}=3.0; 0.50 means 0.50 mg/ml root exudates; 1.00 means 1.00 mg/ml root exudates.

In the clustering result the genes in the same regulons or being functionally related, for example, *iolA, iolB, iolC, iolE, iolF* and *iolG* were clustered in one branch (in the yellow rectangle of Figure 17, also see Appendix Figure 1). Similarly, the genes *dfnF, dfnJ, dfnG,* and *dfnY* were clustered in another subbranch (Figure 18). This suggested a feasibility to predict some genes' functions by means of clustering. To achieve a better prediction, a series of microarray experiments over growth course or under different conditions are usually needed [Eisen et al. 1998]. In this work more caution has to be taken in interpreting the clustering result since only a limited number of experiments were used.



gene	product
ydcD	hypothetical protein YdcD
dfnF	modular polyketide synthase of type I DfnF
ybxF	conserved hypothetical protein YbxF
ylqC	conserved hypothetical protein YlqC
yngL	conserved hypothetical protein YngL
dfnJ	modular polyketide synthase of type I DfnJ
baeI	enoyl-CoA-hydratase BaeI
baeL	polyketide synthase BaeL
citZ	citrate synthase II CitZ
dfnG	modular polyketide synthase of type I DfnG
dfnY	hypothetical protein DfnY
yqzC	hypothetical protein YqzC
RBAM021630	hypothetical protein RBAM021630

Figure 18: A subbranch of genes which were clustered together. The genes *dfnF, dfnJ, dfnG,* and *dfnY*, which are involved in biosynthesis of difficidin, were included in this subbranch.

3.3 Alternative sigma factors, global transcriptional regulators and the response of FZB42 to root Exudates

To further understand the mechanisms of the regulations, seven of FZB42 mutants defective in five sigma factor genes (*sigB, sigD, sigM, sigV,* and *sigX)* and in two transcription regulator genes (*degU* and *abrB*), were included in microarray experiments.

As shown in Table 7, two sets of experiments were performed with all the mutants: I) investigating the transcriptomic response of a mutant to root exudates as performed with FZB42 wild type, that is, comparing the transcriptome of the mutant in the condition of applying root exudates with that of not applying exudates (Mutant+RE<>Mutant-RE); and II) systemically investigating which genes of FZB42 are under the control/regulation of the sigma factors and the transcriptional regulators, that is, comparing the transciptome of a mutant grown in 1CS medium plus root exudates with that of FZB42 wild type grown in the same condition (Mutant+RE<>Wt+RE). A series of data sets were thereby obtained.

To find out the relationship, if there is, between the alternative sigma factors and the regulated genes in response to root exudates, three conditions were applied as described below to screen the genes related to a given sigma factor. When a gene fulfils all the three conditions, i.e., a gene is

1. altered in transcription by root exudates in FZB42 wild type, and
2. directly controlled by a σ factor, namely, down-regulated when the σ factor gene is disrupted,
3. not altered in transcription by root exudates in the σ factor mutant,

it would be proposed that the gene's transcription is affected by root exudates via a mechanism involved by the σ factor.

The same conditions were also applied to the analyses of the transcriptional regulators DegU and AbrB, except a minor modification in the second step: both up-regulated and down-regulated genes were considered as the candidates controlled directly by the regulator, since the DegU and AbrB can either activate or repress the expression of a gene.

The genes meeting the requirement of condition 1 have been discussed in the previous sections (see also Appendix Table 1, Appendix Table 2, and Appendix Table 3). A similar analysis procedure was used to screen the genes which fulfill condition 2 and 3.

3.3.1 Involvement of SigB in the response of FZB42 to root exudates

When the filter condition was set to be q≤0.01 and fold change≥2.0, 29 genes were down-regulated by SigB and thereby identified to be controlled by SigB. Two of the 29 genes (*bmrU* and *csbA*) have previously been reported [Boylan *et al*. 1991; Petersohn *et al*. 1999]. When the same filter condition was applied, 214 genes of the mutant (FZB42 Δ*sigB*) were found to be altered in expression by root exudates and the remaining genes were hence regarded as not altered by root exudates. When these two results were combined with the result of the 302 genes, which were significantly regulated by root exudates in wild type FZB42, two genes were then obtained (Table 13) meeting all the three conditions as defined above. Thereby I propose that genes *glvR* and *pgm1* were regulated by root exudates via the involvement of alternative sigma factor B.

Table 13: The genes proposed to be regulated by root exudates via the involvement of SigB

gene	FCH			product	function involved
	wt+RE<> wt-RE	sigB+RE<> wt+RE	sigB+RE<> sigB-RE		
glvR	4.4	-22.1	#N/A	HTH-type transcriptional regulator GlvR	RNA synthesis
pgm1	2.4	-5.6	#N/A	predicted phosphatase /phosphohexomutase Pgm1	Metabolism of carbohydrates and related molecules

"#N/A" means gene expression was not significantly different (q≤0.01).

3.3.2 Involvement of SigD in the response of FZB42 to root exudates

In order to analyze the genes transcribed by SigD, a comparison of the transcriptome of *sigD* mutant with that of FZB42 wild type were performed in three biological replicates with the cells grown in 1CS medium plus root exudates as was done for σ^B. Besides this, an extra comparison was performed in six biological replicates with the cells grown in 1CS medium without adding root exudates (Table 7). Since the genes controlled by sigD can be identified from both of the comparisons, which were performed in the otherwise same manner except the media and the number of replicates, a comprehensive analysis was conducted based on the two comparisons. In this comprehensive analysis, the filter condition was set to be q≤0.01, FCH≥1.5 in each of the two comparisons, and FCH≥2.0 in at least one of the two comparisons. In this manner 45 genes were found to be down-regulated and then identified to be controlled by SigD, only one of which (*hag*) has previously been reported. However, if a less stringent condition was applied, e.g. q≤0.05 and FCH>1, six and nine genes, which are previously reported to be controlled by SigD,

were found in the two comparisons respectively. Therefore, the filter condition used in this comprehensive analysis was practically rather stringent, in order to reduce as much as possible the number of false positive results.

Using the same condition (q≤0.01, FCH≥2.0) as in the case of SigB, 49 genes were identified to be significantly affected by root exudates in the Δ*sigD* mutant and, accordingly, the other genes were regarded as to have no significant response to root exudates. Taken together, 51 genes fulfilled all the three conditions and were therefore proposed to be regulated in transcription by root exudates via the involvement of σ^D (Table 14). Nineteen of the 51 genes are unknown in function and four of them are unique to *B. amyloliquefaciens* FZB42.

Table 14: The genes proposed to be regulated by root exudates via the involvement of SigD

gene	FCH				function involved
	wt+RE<> wt-RE	sigD<>wt in 1CS	sigD<>wt in 1CS+RE	sigD+RE<> sigD-RE	
amyC	1.8	-2.3	-4.5	#N/A	Transport/binding proteins and lipoproteins
cdd	1.7	-2.0	-2.1	#N/A	Metabolism of nucleotides and nucleic acids
dfnF	2.4	-3.1	-7.8	#N/A	Antibiotic production
dfnI	1.7	-1.9	-4.6	#N/A	Antibiotic production
dfnJ	2.0	-3.8	-11.1	#N/A	Antibiotic production
ebrB	1.8	-2.0	-1.9	#N/A	Transport/binding proteins and lipoproteins
fenE	1.5	-1.5	-2.4	#N/A	Antibiotic production
hag	3.6	-4.1	-27.8	#N/A	Mobility and chemotaxis
lci	-1.6	-6.4	-20.5	#N/A	Antibiotic production
luxS	1.7	-1.7	-2.1	#N/A	Sensors (signal transduction)
rapA	1.7	-3.4	-7.2	#N/A	Sporulation
RBAM00715	-1.7	-8.9	-11.9	#N/A	Transport/binding proteins and lipoproteins
RBAM01763	-2.0	-3.6	-2.6	#N/A	unknown
RBAM01835	-1.6	-3.6	-3.2	#N/A	unknown_ No similarity
RBAM03224	-1.6	-1.9	-2.3	#N/A	unknown_ No similarity
RBAM03561	1.8	-10.0	-37.6	#N/A	unknown_ No similarity
RBAM03844	-1.8	-2.5	-2.3	#N/A	unknown_ No similarity
resA	1.7	-2.1	-1.8	#N/A	Membrane bioenergetics
rplM	1.8	-1.6	-2.5	#N/A	Protein synthesis
rpsM	1.6	-2.3	-1.6	#N/A	Protein synthesis
rpsR	2.1	-2.0	-2.1	#N/A	Protein synthesis
rpsU	3.1	-2.3	-4.6	#N/A	Protein synthesis
scoB	1.6	-2.0	-2.4	#N/A	Metabolism of lipids
sda	1.7	-1.5	-7.8	#N/A	Sporulation
secE	1.7	-2.2	-3.8	#N/A	Protein secretion
sigW	2.4	-2.9	-3.7	#N/A	RNA synthesis
spoIIB	-1.7	-2.0	-1.5	#N/A	Sporulation
srfAD	1.9	-1.5	-2.1	#N/A	Antibiotic production
sucC	1.9	-1.6	-2.1	#N/A	Metabolism of carbohydrates and related molecules
yabR	1.7	-1.7	-3.2	#N/A	Metabolism of nucleotides and nucleic acids

ybbM	3.2	-1.8	-3.7	#N/A	RNA synthesis
ybxF	2.0	-2.1	-1.8	#N/A	Protein synthesis
ydcD	2.2	-2.8	-4.9	#N/A	unknown
ydeB	2.9	-2.6	-2.1	#N/A	RNA synthesis
yfiT	1.5	-1.9	-3.0	#N/A	unknown
yheA	1.7	-2.2	-1.5	#N/A	unknown
yisK	1.6	-1.6	-2.6	#N/A	Metabolism of amino acids and related molecules
ylbN	1.6	-2.1	-2.5	#N/A	unknown
yllB	2.1	-3.5	-3.5	#N/A	unknown
ylqC	1.8	-2.0	-1.7	#N/A	unknown
ymcA	2.5	-4.2	-3.0	#N/A	unknown
yngL	2.0	-1.8	-2.6	#N/A	unknown
ypmP	2.2	-2.1	-2.1	#N/A	unknown
yppF	1.5	-2.2	-2.5	#N/A	unknown
ytxG	1.5	-1.5	-2.1	#N/A	Adaptation to atypical conditions
yukE	1.7	-4.4	-3.8	#N/A	unknown
yusL	1.6	-2.8	-1.8	#N/A	Metabolism of lipids
yvqI	1.5	-2.9	-2.3	#N/A	unknown
yvyD	1.8	-3.3	-2.1	#N/A	RNA synthesis
ywcI	-4.0	-5.9	-4.2	#N/A	unknown
yxjC	1.9	-1.8	-2.9	#N/A	unknown

"#N/A" means gene expression was not significantly different (q≤0.01).

3.3.3 Involvement of ECF sigma factors in the response of FZB42 to root exudates

The three ECF sigma factor ($\sigma^{M,}$ σ^{V} and σ^{X}) mutants were studied in the same way for their involvement in response to root exudates. The analysis condition (q≤0.01 and FCH≥2.0) and procedures were exactly the same as for SigB. The results are shown in Table 15, Table 16 and Table 17. Only four genes were proposed to be altered in transcription by root exudates via the involvement of σ^{V}, while 15 and 22 genes were altered via σ^{M} and σ^{V}, respectively. Regarding those affected by σ^{X}, 10 out of the 22 genes resided in an operon cluster related to protein synthesis or secretion, as highlighted in Table 17.

Table 15: The genes proposed to be regulated by root exudates via the involvement of SigM

gene	FCH			function involved
	wt+RE<> wt-RE	sigM+RE <>wt+RE	sigM+RE<> sigM-RE	
comS	1.7	-4.0	#N/A	Transformation/competence
yngL	2.0	-3.8	#N/A	unknown
spoIIID	-1.5	-3.5	#N/A	RNA synthesis
yllB	2.1	-2.6	#N/A	unknown

spoIIB	-1.7	-2.6	#N/A	Sporulation
hrcA	1.9	-2.3	#N/A	RNA synthesis
ycsD	1.8	-2.2	#N/A	Metabolism of lipids
yurL	-1.5	-2.2	#N/A	Metabolism of amino acids and related molecules
srfAD	1.9	-2.1	#N/A	Antibiotic production
med	-1.6	-2.1	#N/A	Transformation/competence
yqeW	-1.5	-2.1	#N/A	Transport/binding proteins and lipoproteins
yurP	-1.9	-2.0	#N/A	Metabolism of amino acids and related molecules
ywcI	-4.0	-2.0	#N/A	Unknown
ylbK	-1.6	-2.0	#N/A	unknown
yfjT	-1.8	-2.0	#N/A	unknown

"#N/A" means gene expression was not significantly different (q≤0.01).

Table 16: The genes proposed to be regulated by root exudates via the involvement of SigV

gene	FCH			function involved
	wt+RE<> wt-RE	sigV+RE <>wt+RE	sigV+RE<> sigV-RE	
ywqB	-1.6	-4.2	#N/A	Unknown
yurP	-1.9	-2.7	#N/A	Metabolism of amino acids and related molecules
med	-1.6	-2.5	#N/A	Transformation/competence
yfjT	-1.8	-2.3	#N/A	Unknown

"#N/A" means gene expression was not significantly different (q≤0.01).

Table 17: The genes proposed to be regulated by root exudates via the involvement of SigX

gene	FCH			function involved
	wt+RE<> wt-RE	sigX+RE <>wt+RE	sigX+RE<> sigX-RE	
atpC	1.6	-2.5	#N/A	Membrane bioenergetics
flgM	1.7	-2.4	#N/A	Mobility and chemotaxis
fliM	2	-2.2	#N/A	Mobility and chemotaxis
fusA	2.2	-2.7	#N/A	Protein synthesis
hag	3.6	-3.5	#N/A	Mobility and chemotaxis
infA	2	-4.3	#N/A	Protein synthesis
map	3.1	-2.3	#N/A	Protein modification
rplA	1.7	-2.5	#N/A	Protein synthesis
rplD	1.8	-2.1	#N/A	Protein synthesis
rplJ	2	-2.2	#N/A	Protein synthesis
rpoA	2	-2.4	#N/A	RNA synthesis
rpoC	1.9	-2.4	#N/A	RNA synthesis
rpsK	1.6	-2.1	#N/A	Protein synthesis
rpsM	1.6	-2.8	#N/A	Protein synthesis
rpsR	2.1	-2	#N/A	Protein synthesis
secY	2	-2.4	#N/A	Protein secretion
ssb	1.6	-2.2	#N/A	DNA replication
sucD	1.7	-2.5	#N/A	Metabolism of carbohydrates and related molecules

tufA	1.5	-2.1	#N/A	Protein synthesis
veg	2.8	-2	#N/A	Miscellaneous
yabP	2.1	-3.7	#N/A	unknown
yjbD	1.5	-2	#N/A	unknown

"#N/A" means gene expression was not significantly different (q≤0.01).

3.3.4 Involvement of AbrB in the response of FZB42 to root exudates

The disruption of *abrB* and *degU* could not only positively but also negatively affect the expression of genes. Therefore the identification of genes controlled by them included those which were both up-regulated and down-regulated. Except this step, the procedures used to find genes for AbrB meeting the three conditions were the same as in the case of SigB. As a result, 149 genes were proposed to be, via the involvement of AbrB, altered in transcription in response to root exudates (Table 18). Although the molecular mechanisms of regulation of AbrB in transcription remain unclear, the number of genes affected by this regulator was the largest among the seven transcriptional factors investigated in this study.

Table 18: The genes proposed to be regulated by root exudates via the involvement of AbrB

gene	***FCH***			***function involved***
	wt+RE<> wt-RE	***abrB+RE <>wt+RE***	***abrB+RE<> abrB-RE***	
ftsL	1.7	4.1	#N/A	Cell division
ftsH	1.5	5.3	#N/A	Cell division
qoxA	1.6	2.4	#N/A	Membrane bioenergetics
atpF	1.5	2.8	#N/A	Membrane bioenergetics
qoxB	1.6	3.1	#N/A	Membrane bioenergetics
atpH	1.7	6.3	#N/A	Membrane bioenergetics
atpC	1.6	14.3	#N/A	Membrane bioenergetics
yjlD	1.6	32.9	#N/A	Membrane bioenergetics
fliP	1.7	-2.9	#N/A	Mobility and chemotaxis
hag	3.6	3.4	#N/A	Mobility and chemotaxis
cheC	1.7	3.5	#N/A	Mobility and chemotaxis
lytA	1.5	2.1	#N/A	Protein secretion
tatAy	1.6	2.5	#N/A	Protein secretion
secY	2.0	12.9	#N/A	Protein secretion
secE	1.7	34.7	#N/A	Protein secretion
luxS	1.7	4.1	#N/A	Sensors (signal transduction)
sda	1.7	2.9	#N/A	Sporulation
comS	1.7	6.1	#N/A	Transformation/competence
gutA	2.8	-10.5	#N/A	Transport/binding proteins and lipoproteins
araQ	1.9	-2.5	#N/A	Transport/binding proteins and lipoproteins
rocE	4.0	-2.1	#N/A	Transport/binding proteins and lipoproteins
ytmK	1.6	2.0	#N/A	Transport/binding proteins and lipoproteins
yufN	1.7	2.0	#N/A	Transport/binding proteins and lipoproteins
mscL	1.8	3.4	#N/A	Transport/binding proteins and lipoproteins

ykqB	1.6	4.4	#N/A	Transport/binding proteins and lipoproteins
oppD	1.5	5.4	#N/A	Transport/binding proteins and lipoproteins
RBAM00714	-1.5	7.6	#N/A	Transport/binding proteins and lipoproteins
RBAM00715	-1.7	76.9	#N/A	Transport/binding proteins and lipoproteins
recA	1.6	16.8	#N/A	DNA recombination
ssb	1.6	8.7	#N/A	DNA replication
rpsI	1.7	3.1	#N/A	Protein synthesis
rplD	1.8	4.6	#N/A	Protein synthesis
rplU	2.0	5.0	#N/A	Protein synthesis
rpsK	1.6	5.1	#N/A	Protein synthesis
rpmA	1.6	5.5	#N/A	Protein synthesis
rplM	1.8	5.5	#N/A	Protein synthesis
fusA	2.2	6.9	#N/A	Protein synthesis
rpsO	1.6	7.4	#N/A	Protein synthesis
rpsU	3.1	8.1	#N/A	Protein synthesis
rpsR	2.1	8.6	#N/A	Protein synthesis
tufA	1.5	10.8	#N/A	Protein synthesis
rplJ	2.0	18.2	#N/A	Protein synthesis
rpmGA	1.7	145.6	#N/A	Protein synthesis
rplA	1.7	210.5	#N/A	Protein synthesis
trmU	1.5	10.2	#N/A	RNA modification
glvR	4.4	-129.7	#N/A	RNA synthesis
spoIIID	-1.5	-6.2	#N/A	RNA synthesis
glpP	1.8	3.3	#N/A	RNA synthesis
rpoA	2.0	3.8	#N/A	RNA synthesis
fapR	1.5	4.6	#N/A	RNA synthesis
yvyD	1.8	5.7	#N/A	RNA synthesis
phoP	1.9	6.9	#N/A	RNA synthesis
ydeB	2.9	7.2	#N/A	RNA synthesis
rpoC	1.9	9.2	#N/A	RNA synthesis
iolA	2.7	-3.8	#N/A	Metabolism of amino acids and related molecules
yurP	-1.9	-2.0	#N/A	Metabolism of amino acids and related molecules
rocD	6.5	-2.0	#N/A	Metabolism of amino acids and related molecules
ansA	1.6	3.6	#N/A	Metabolism of amino acids and related molecules
gudB	1.5	3.6	#N/A	Metabolism of amino acids and related molecules
cysC	1.5	3.7	#N/A	Metabolism of amino acids and related molecules
gcvPB	1.6	4.0	#N/A	Metabolism of amino acids and related molecules
gcvT	1.8	6.6	#N/A	Metabolism of amino acids and related molecules
lacG	2.7	-141.0	#N/A	Metabolism of carbohydrates and related molecules
glvA	5.2	-129.1	#N/A	Metabolism of carbohydrates and related molecules
galT1	4.2	-45.0	#N/A	Metabolism of carbohydrates and related molecules
lacE	1.6	-25.9	#N/A	Metabolism of carbohydrates and related molecules
lacF	1.8	-17.1	#N/A	Metabolism of carbohydrates and related molecules
pgm1	2.4	-9.3	#N/A	Metabolism of carbohydrates and related molecules
araB	1.7	-4.2	#N/A	Metabolism of carbohydrates and related molecules
araL	2.6	-3.2	#N/A	Metabolism of carbohydrates and related molecules
araM	2.3	-2.6	#N/A	Metabolism of carbohydrates and related molecules
iolB	2.7	-2.5	#N/A	Metabolism of carbohydrates and related molecules
acoL	1.5	2.1	#N/A	Metabolism of carbohydrates and related molecules
ycsN	1.6	2.8	#N/A	Metabolism of carbohydrates and related molecules
citB	1.7	3.4	#N/A	Metabolism of carbohydrates and related molecules

iolS	1.7	3.6	#N/A	Metabolism of carbohydrates and related molecules
citZ	2.3	3.9	#N/A	Metabolism of carbohydrates and related molecules
glpK	1.5	4.3	#N/A	Metabolism of carbohydrates and related molecules
sucD	1.7	4.4	#N/A	Metabolism of carbohydrates and related molecules
mdh	1.9	6.4	#N/A	Metabolism of carbohydrates and related molecules
rpe	1.5	6.8	#N/A	Metabolism of carbohydrates and related molecules
pgm2	1.8	2.7	#N/A	Metabolism of carbohydrates and related molecules
pgi	1.5	6.8	#N/A	Metabolism of carbohydrates and related molecules
sucC	1.9	10.5	#N/A	Metabolism of carbohydrates and related molecules
gapB	1.6	31.4	#N/A	Metabolism of carbohydrates and related molecules
ywkE	1.6	2.7	#N/A	Metabolism of coenzymes and prosthetic groups
hepT	2.0	5.6	#N/A	Metabolism of coenzymes and prosthetic groups
ycsD	1.8	-2.0	#N/A	Metabolism of lipids
ptb	1.7	2.2	#N/A	Metabolism of lipids
yusL	1.6	2.3	#N/A	Metabolism of lipids
ydbM	1.5	2.4	#N/A	Metabolism of lipids
bcd	1.8	6.7	#N/A	Metabolism of lipids
bkdAA	1.7	7.4	#N/A	Metabolism of lipids
nin	1.5	-2.0	#N/A	Metabolism of nucleotides and nucleic acids
yabR	1.7	2.0	#N/A	Metabolism of nucleotides and nucleic acids
cdd	1.7	2.6	#N/A	Metabolism of nucleotides and nucleic acids
pyrH	1.5	2.7	#N/A	Metabolism of nucleotides and nucleic acids
yvgQ	1.5	6.6	#N/A	Metabolism of sulfur
ytxG	1.5	9.3	#N/A	Adaptation to atypical conditions
mlnH	1.5	2.0	#N/A	Antibiotic production
baeE	1.6	2.0	#N/A	Antibiotic production
srfAD	1.9	2.4	#N/A	Antibiotic production
difG	2.0	2.5	#N/A	Antibiotic production
baeN	1.5	3.5	#N/A	Antibiotic production
srfAC	1.7	3.9	#N/A	Antibiotic production
baeR	2.3	3.9	#N/A	Antibiotic production
difJ	2.0	5.6	#N/A	Antibiotic production
difI	1.7	6.5	#N/A	Antibiotic production
lci	-1.6	16.8	#N/A	Antibiotic production
yceF	1.7	3.1	#N/A	Detoxification
yceE	1.8	4.2	#N/A	Detoxification
era	2.2	2.2	#N/A	Miscellaneous
yurV	1.7	3.1	#N/A	Miscellaneous
veg	2.8	5.6	#N/A	Miscellaneous
xhlA	1.6	8.7	#N/A	Phage-related functions
RBAM02992	1.6	2.1	#N/A	Unknown
RBAM01886	1.5	3.0	#N/A	Unknown
RBAM01835	-1.6	3.5	#N/A	Unknown
RBAM00434	2.5	20.9	#N/A	Unknown
RBAM03844	-1.8	24.2	#N/A	Unknown
yqjL	1.5	-2.5	#N/A	Unknown
yfjT	-1.8	-2.0	#N/A	Unknown
ycgB	1.5	2.1	#N/A	Unknown
ykqC	1.6	2.3	#N/A	Unknown
yqkC	1.8	2.7	#N/A	Unknown
ypiB	2.0	2.7	#N/A	Unknown

yjlC	1.5	2.9	#N/A	Unknown
ylbN	1.6	3.0	#N/A	Unknown
yhjN	1.5	3.0	#N/A	Unknown
yqeY	2.5	3.0	#N/A	Unknown
yoeB	1.6	3.1	#N/A	Unknown
ypeP	1.5	3.3	#N/A	Unknown
ymcA	2.5	3.5	#N/A	Unknown
ywlA	1.6	3.8	#N/A	Unknown
yaaR	1.6	4.5	#N/A	Unknown
yrdA	1.8	4.8	#N/A	Unknown
ydcE	1.5	4.8	#N/A	Unknown
ylqC	1.8	5.6	#N/A	Unknown
yqhY	1.5	5.7	#N/A	Unknown
yngL	2.0	5.8	#N/A	Unknown
yukE	1.7	6.6	#N/A	Unknown
ymcB	2.1	6.6	#N/A	Unknown
yqxD	1.5	6.8	#N/A	Unknown
engC	1.8	11.8	#N/A	Unknown
yqzC	1.7	12.2	#N/A	Unknown
yjbD	1.5	27.0	#N/A	Unknown
RBAM00435	1.7	2.3	#N/A	Unknown
RBAM03268	1.9	4.1	#N/A	Unknown
RBAM01042	1.6	4.2	#N/A	Unknown

"#N/A" means gene expression was not significantly different (q≤0.01).

3.3.5 Involvement of DegU in the response of FZB42 to root exudates

Similar to the *sigD*$^{-}$ mutant, another three replicates were performed with the cells grown in 1CS medium without root exudates, in order to indentify genes regulated by DegU. Therefore, the analysis procedures applied to DegU was the same as those to SigD. In this way, 128 genes were identified to be regulated by DegU, four of which (*comK*, *degQ*, *nprE* and *ispA*) have previously been reported. Satisfying the three conditions, 39 genes (Table 19) were finally proposed to be altered in transcription by root exudates via the involvement of DegU. One third of the 39 genes are unknown in function. The other genes with known function are involved in various biological aspects, which reflects a pleiotropic regulation of DegU in post-exponential phase.

All transcriptional factors involved in response of the 302 genes to root exudates were summarized in Appendix Table 1, Appendix Table 2, and Appendix Table 3. Although further confirmations are necessary, this study provides a systematic investigation suggesting the mechanisms of how the genes, which were significantly altered in expression, of *B. amyloliquefaciens* FZB42 were regulated in response to root exudates.

Table 19: The genes proposed to be regulated by root exudates via the involvement of DegU

Gene	Fch				function involved
	wt+RE<> wt-RE	*sigD<>wt in 1CS*	*sigD<>wt in 1CS+RE*	*sigD+RE<> sigD-RE*	
resA	1.7	-2	-1.6	#N/A	Membrane bioenergetics
hag	3.6	6.3	1.5	#N/A	Mobility and chemotaxis
rapA	1.7	3.3	2	#N/A	Sporulation
comS	1.7	8.8	4.6	#N/A	Transformation/competence
cimH	1.6	-1.6	-2.9	#N/A	Transport/binding proteins and lipoproteins
oppD	1.5	2.1	1.6	#N/A	Transport/binding proteins and lipoproteins
oppF	1.6	2.5	1.7	#N/A	Transport/binding proteins and lipoproteins
ytnA	1.9	2.5	1.7	#N/A	Transport/binding proteins and lipoproteins
yufN	1.7	3.9	2.9	#N/A	Transport/binding proteins and lipoproteins
yxaL	1.5	3	2.6	#N/A	Protein modification
rpmGA	1.7	-2.5	-4.2	#N/A	Protein synthesis
RBAM00542	-1.7	-2.7	-1.7	#N/A	RNA synthesis
sigW	2.4	3.7	1.8	#N/A	RNA synthesis
ybbM	3.2	5.5	1.9	#N/A	RNA synthesis
gudB	1.5	-1.5	-2.4	#N/A	Metabolism of amino acids and related molecules
glvA	5.2	-2.2	-27.8	#N/A	Metabolism of carbohydrates and related molecules
ycsN	1.6	2.2	1.6	#N/A	Metabolism of carbohydrates and related molecules
scoB	1.6	2.3	1.8	#N/A	Metabolism of lipids
cdd	1.7	2.1	1.5	#N/A	Metabolism of nucleotides and nucleic acids
pyrF	-1.6	2.2	2.7	#N/A	Metabolism of nucleotides and nucleic acids
yabR	1.7	2.3	1.5	#N/A	Metabolism of nucleotides and nucleic acids
degR	1.5	3.5	2.5	#N/A	Adaptation to atypical conditions
fenE	1.5	-1.7	-2.1	#N/A	Antibiotic production
srfAC	1.7	6.8	3.9	#N/A	Antibiotic production
srfAD	1.9	6.8	4.1	#N/A	Antibiotic production
era	2.2	2.9	1.8	#N/A	Miscellaneous
RBAM00434	2.5	-13.6	-50.8	#N/A	unknown_ No similarity
RBAM01835	-1.6	-2.9	-1.9	#N/A	unknown_ No similarity
RBAM03224	-1.6	3.1	6.1	#N/A	unknown_ No similarity
RBAM03561	1.8	-4.9	-10.5	#N/A	unknown_ No similarity
ydcD	2.2	2.7	1.7	#N/A	unknown
yllB	2.1	2.5	1.5	#N/A	unknown
yngL	2	-1.9	-2.6	#N/A	unknown
ypmP	2.2	3.3	1.6	#N/A	unknown
yqeZ	2	2.7	1.8	#N/A	unknown
yukE	1.7	-7.5	-12.4	#N/A	unknown
yvqI	1.5	5.8	2.9	#N/A	unknown
ywcI	-4	-8.4	-2	#N/A	unknown
RBAM01763	-2	1.5	3	#N/A	unknown

"#N/A" means the gene was not significantly differentially (q≤0.01) expressed.

3.4 sRNAs involved in the response of FZB42 to root exudates

The presence of small regulatory RNAs in *B. amyloliquefaciens* has not been studied so far, although some have been identified in the closely related *B. subtilis* [Saito *et al*. 2009]. Due to their advantages in gene regulations, small RNAs may play an important role in plant-bacteria interactions. In this work a comparative genomics-based screen for candidate sRNAs in *B. amyloliquefaciens* FZB42 were performed. Passing the stringency applied, 238 hits were found in the intergenic regions of FZB42 genome.

With the condition of $q \leq 0.01$ and $FCH \geq 1.5$, the analysis of six biological replicates suggested that in total 20 sRNA candidates (Table 20) were significantly altered in expression at OD3.0 by root exudates, while none was affected at OD1.0.

Table 20: sRNA candidates that were differentially expressed in response to root exudates

Name	FCH	upstream	Downstream
Igr3849	1.6	174 bp at 5' side: TyrS	94 bp at 3' side: AcsA
Igr3873	-1.6	61 bp at 5' side: PanB	115 bp at 3' side: BirA
Igr3893	1.8	136 bp at 5' side: hypothetical protein	76 bp at 3' side: hypothetical protein
Igr3906	1.8	355 bp at 5' side: rRNA-16S ribosomal RNA	143 bp at 3' side: YuaJ
Igr3925	1.8	30 bp at 5' side: YjdF	321 bp at 3' side: YtwI
Igr3927	2.4	145 bp at 5' side: PolA	33 bp at 3' side: PhoR
Igr3931	1.8	12 bp at 5' side: InfC	309 bp at 3' side: YsbB
Igr3959	1.6	185 bp at 5' side: RecO	81 bp at 3' side: Era
Igr4023	-1.5	241 bp at 5' side: hypothetical protein	36 bp at 3' side: RtpA
Igr4026	2.2	49 bp at 5' side: TruA	42 bp at 3' side: RplM
Igr4028	1.9	57 bp at 5' side: RpsK	51 bp at 3' side: RpoA
Igr3817	1.6	78 bp at 5' side: SpeD	86 bp at 3' side: GapB
Igr3839	-1.5	65 bp at 5' side: NusG	34 bp at 3' side: RplK
Igr3941	1.8	50 bp at 5' side: RplU	27 bp at 3' side: SpoIVFB
Igr3947	-1.5	114 bp at 5' side: YrvM	143 bp at 3' side: AspS
Igr3840	1.6	56 bp at 5' side: NusG	43 bp at 3' side: RplK
Igr3832	1.6	121 bp at 5' side: hypothetical protein	30 bp at 3' side: CspC
Igr3952	1.6	118 bp at 5' side: YrhF	75 bp at 3' side: YrhE
Igr4016	1.5	99 bp at 5' side: YdeH	101 bp at 3' side: hypothetical protein
Igr4030	2.5	55 bp at 5' side: YbxF	12 bp at 3' side: RpsL

However, the sRNA candidates found *in silico* still needed to be confirmed experimentally for their virtual existence. Northern blot is a routine approach to detect small RNAs. Oligonucleotide probes with a complementary sequence to the 20 candidates were therefore designed and labeled with ^{32}P for Northern blot. As illustrated in Figure 19 and Table 21, the hybridization result verified the transcripts of six sRNA candidates (Igr3873, Igr3906, Igr3927, Igr3931, Igr3959, Igr4026 and Igr4028). Nevertheless, caution and further confirmation need to be paid to Igr4026 (Figure 19, panel F), which showed only a weak band in Northern blot.

Table 21: sRNAs identified by Northern blot

name	FCH (microarray)	FCH[a] (Northern Blot)	Left gene (length_direction)	Length (nt)	Orientation	Right gene (length- direction)
Igr3906	1.8	-2.5	rrnA-J-16S (1.55kb_<<<)	~170	>>>	yuaJ (582bp_>>>)
Igr3927	2.4	---	polA (2.64kb_<<<)	~60	>>>	phoR (1.714b_<<<)
Igr3931	1.8	-5.3	infC (504bp_<<<)	~140	<<<	ysbB (684bp_<<<)
Igr3959	1.6	1.3	recO (768bp_<<<)	~340	<<<	era (906bp_<<<)
Igr4026	2.2	3.5	truA (744bp_>>>)	~190	>>>	rplM (438bp_>>>)
Igr4028	2.0	1.8	rpsK (396bp_>>>)	~320	>>>	rpoA (945bp_>>>)

Remarks: a: the RNA samples used were the same as those used in microarray experiments; --: Igr3927 disappeared completely in the presence of root exudates; nt: nucleotide

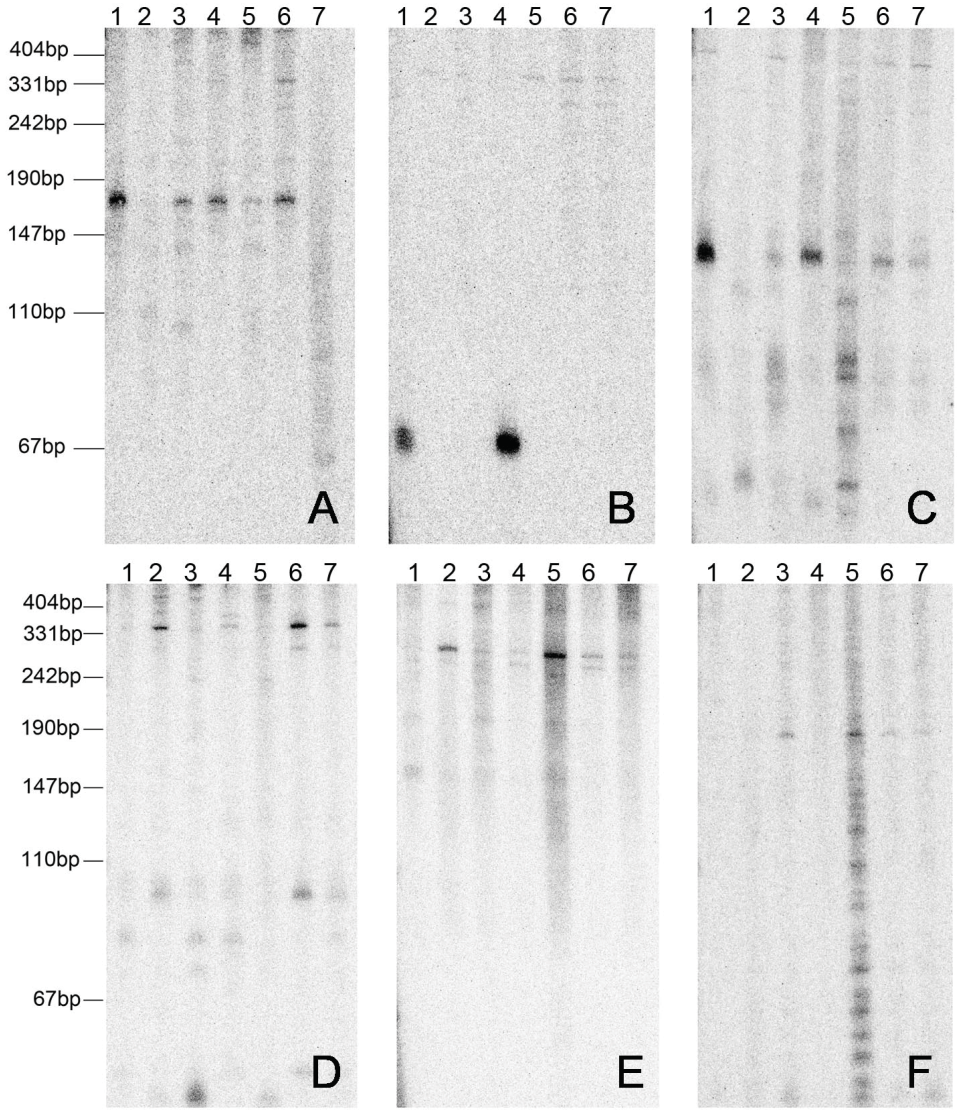


Figure 19: Identification of six sRNA candidates by means of Northern blot.

A: Igr3906; B: Igr3927; C: Igr3931; D: Igr3959; E: Igr4028; F: Igr4026; 1: 24°C_OD3.0-RE; 2: 24°C_OD1.0+RE; 3: 24°C_OD3.0+RE; 4: 37°C_OD3.0-RE; 5: 37°C_OD1.0+RE; 6: 37°C_OD2.0+RE; 7: 37°C_OD3.0+RE.

3.4.1 Responses of sRNAs to the root exudates

In this work Northern blot was employed not only to detect the existence of sRNAs but also to confirm the microarray result concerning the responses of the sRNAs to root exudates. To facilitate further work and to increase detection rate, RNA samples collected at 37°C from three growth phases (OD1.0, OD2.0 and OD3.0, respectively) were also included in Northern blot, together with the RNA samples used in the microarray

experiments, which were obtained at 24°C and from two growth phases (OD1.0 and OD3.0 respectively).

Three out of the six sRNAs showed a discrepancy between the Northern blot result and microarray result in terms of their response to the root exudates (Figure 20). The transcripts of Igr3906, Igr3927 and Igr3931 decreased obviously at 24°C/OD3.0 in response to root exudates, especially Igr3927, which was completely extinguished. The transcripts of them displayed a similar result at 37°C/OD3.0. We think the result of Northern blot more closely reflected the reality because of several reasons. Firstly, Northern blot adopted less experimental procedures than microarray, thus reducing the bias or system errors which may be introduced. Secondly, Northern blotting adopted more amount of total RNAs, which therefore provided a more reliable, although maybe less sensitive, quantative method than microarray. Finally, in Northern blot each oligonucleotide probe was specifically devoted to detecting one sRNA, while in microarray experiments thousands of reverse-transcribed cDNAs were competitively hybridized with the probes on a chip. This competition between cDNAs would greatly influence their hybridization efficiency with sRNA probes, especially when taking into consideration that sRNA sequences have a short length and a strong secondary structure-forming tendency.

The other three sRNAs (Igr3959, Igr4026 and Igr4028) presented a consistent response with what was obtained in microarray results, although OD3.0 was not an optimal sampling point where sRNAs are expected to express abundantly. This fact implies a limitation of the two-color microarray system used that only the relative ratio of a gene's expression in one sample to that in another sample to be compared was emphasized while there is no practically acceptable way to quantify the absolute expression of the genes.

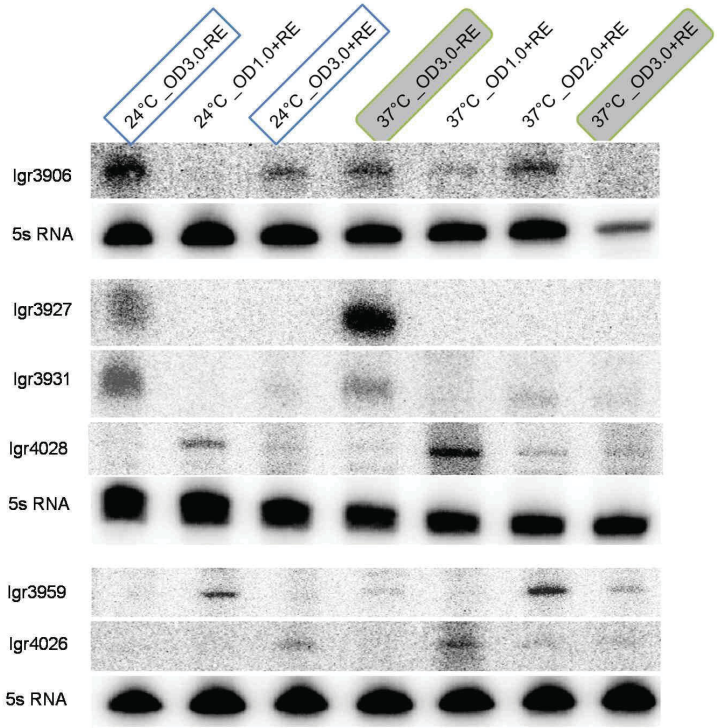


Figure 20: Responses of the six sRNAs to the root exudates

3.4.2 Effects of the alternative σ factors, AbrB and DegU on the sRNAs

The effects of the alternative σ factors and the transcriptional regulators, AbrB and DegU, on the expression of the sRNA genes were similarly profiled as described above for the protein-coding genes. Using the same condition, none of the alternative σ factors was found to be the possible transcription factor involved in response to root exudates. However, the up-regulated expression of Igr4026 by root exudates was further supported by the evidence that the Igr4026 gene in the mutants (FZB42 Δ*sigB*, and FZB42 Δ*sigX*) showed the same enhanced transcription in response to root exudates as in FZB42 wild type.

Unlike the sigma factors, AbrB and DegU were shown to regulate the transcription of five sRNAs and affect the responses of two sRNAs to root exudates, as shown in Table 22. It has been reported that sRNA BsrF is activated by the global regulator CodY in the presence of branched-chain amino acid and GTP [Preis *et al*. 2009], however, to our best knowledge, this is the first suggestion that AbrB and DegU are involved in expression of sRNAs.

Table 22: The effects of AbrB and DegU on the expression of sRNAs

sRNA	FCH				
	wt+RE<>wt-RE	degU+RE<>wt+RE	degU+RE<>degU-RE	abrB+RE<>wt+RE	abrB+RE<>abrB-RE
Igr3906	1.8	3.0	#N/A	#N/A	#N/A
Igr3927	2.4	#N/A	#N/A	3.0	#N/A
Igr3931	1.8	#N/A	#N/A	4.9	-2.3
Igr3959	1.6	2.0	#N/A	3.6	#N/A
Igr4026	2.2	#N/A	#N/A	6.8	-2.4
Igr4028	1.9	#N/A	#N/A	#N/A	#N/A

"#N/A" means the sRNA was not significantly differentially (q≤0.01) expressed.

3.4.3 Characterization of the six sRNAs identified

Igr3906 was experimentally confirmed in this work for the first time. Based on multiple alignments, the Igr3906 sequence is conserved in phylogenetically related species such as *B. subtilis, B. pumilus,* and *B. licheniformis*. The counterpart of Igr4026 in *B. subtilis* was annotated as non-coding small RNA BSU_misc_RNA_51, a possible TPP riboswitch, which binds directly to thiamine pyrophosphate (TPP) to regulate the expression of a variety of genes, mostly transporters [Miranda-Rios *et al*. 2001; Rodionov *et al*. 2002].

Igr3927 has a quite short sequence of approximately 60 nucleotides (Figure 19 or Figure 20). Igr3927 is also highly conserved in the related species but it has not been annotated so far. The structure of Igr3927 predicted by RNA mfold [Zuker 2003] displays a typical stem-loop secondary structure with a ploy-U tail (Figure 21). It is intriguing that the expression of Igr3927 was nearly completely repressed by the addition of root exudates (Figure 19 or Figure 20), at both 24°C and 37°C.

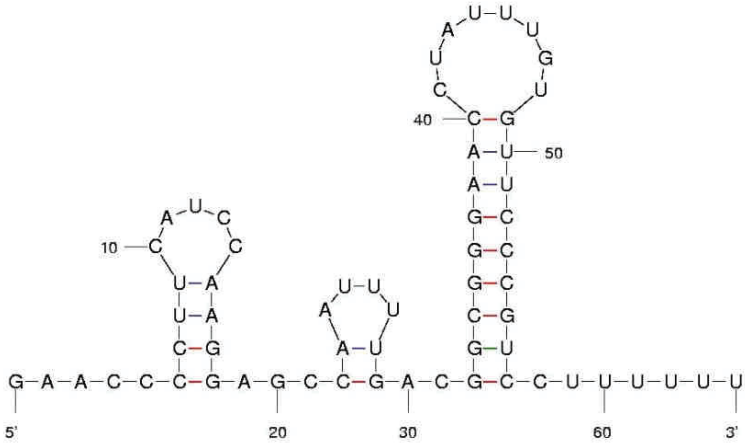


Figure 21: The predicted structure of sRNA Igr3927

The counterpart of Igr3931 in *Bacillus subtilis* was annotated as BSU_misc_RNA_47, which is a putative ribosomal protein leader found in *B. subtilis* and other low-GC Gram-positive bacteria [Zengel *et al*. 1994]. It is an autoregulatory structure located in the 5'-UTR of mRNA encoding for translation initiation factor IF-3 followed by ribosomal proteins L35 and L20 (*infC-rpmI-rplT*). The transcription of Igr3931 was also strongly inhibited by root exudates.

The intergenic region between *recO* and *era*, where Igr3959 resides, has now been annotated as *yqzL* encoding a hypothetical protein with unknown function [Barbe *et al*. 2009]. Since dual function RNAs which not only perform base paring-dependent regulation but also encode a polypeptide have been reported [Boisset *et al*. 2007; Wadler *et al*. 2007], we do not exclude Igr3959 to function as a sRNA.

Both Igr4026 and Igr4028 have a sequence longer than the intergenic regions where they reside: Igr4026 is, according to Northern blot, around 190 nt while the intergenic region between *truA* and *rplM* is only 159 bp; likewise, Igr4028 is displayed to be around 320 nt in Northern blot while the intergenic region between *rpsK* and *rpoA* is only 176 bp. This indicates that Igr4026 and Igr4028 are probably *cis*-acting elements, which regulate the expression of their neighboured genes.

4 Discussion

4.1 Plant colonization by *B. amyloliquefaciens* FZB42

In this work the labeling of *B. amyloliquefaciens* FZB42 with several fluorescent proteins by chromosomal integration and the specific colonization patterns of GFP-labeled FZB42 cells on three different kinds of plants in a gnotobiotic system have been described.

4.1.1 Fluorescent protein-labeling of FZB42

The labeling of FZB42 work was performed by integrating a copy of GFP gene on the bacterial chromosome instead of episomic tagging with plasmid-borne GFP, which is used in many cases. Foreign plasmids containing *gfp* are often unstable in *Bacilli*, suffering from loss of plasmids in replication or fluctuation of GFP expression. By contrast, chromosomal integration can endue bacterial cells with more stable and uniform fluorescence, which would significantly favor a later colonization study. However, low fluorescence intensity could be a potential disadvantage of this method because only a single copy of *gfp* was introduced. This would be problematic for microscopic observation, especially taking into account that G^+ bacteria possess a thicker cell wall. This concern seemed to be true with the GFP-labeled FB01 cells, whose fluorescence was not very bright and was significantly photobleached within 10 seconds. In order to increase the brightness and/or the photostability, several methods were tried including replacing the P_{spac} promoter with two indigenous promoters of FZB42 and using various suspension buffers for specimen preparation; however, all of these attempts were not helpful.

The brighter fluorescence of FB01mut greatly facilitated microscopic observations, although it is not superior to FB01 in terms of photostability. Surprisingly, the FB01mut cells colonizing plants were remarkably more resistant to photobleaching than those grown on LB agar. Then there was no any obstacle in observations, especially when scanned with CLSM. It is almost certain that the improved tolerance was attributed to the specific microenvironments of their habitats on plant roots. This phenomenon revealed a significant effect of biological processes of plants on their associated rhizobacteria.

The idea behind labeling FZB42 with red fluorescent protein was to make it possible that FZB42 wild type and its mutants could be tagged with different fluorescent colors and then allow to be specifically recovered from plants roots, e.g. by FACS sorting, for a

subsequent transcriptomic investigation. However, DsRed turned out not to be a good tag due to its relatively weak brightness and obvious cell-to-cell fluorescence variations. As an improved derivative of DsRed, TdTomato was also evaluated for labeling. Although TdTomato-labeled cells had a better performance at 37°C than DsRed-labeled ones, they were still not so suitable as GFP-labeled cells for a plant colonization experiment.

4.1.2 Colonization patterns by FZB42 on three plants

The junctions between primary roots and lateral roots were found to be a favored habitat of FZB42, consistent with results obtained with *Pseudomonas* colonization. In additioin, root hairs were another preferred position by FZB42. This phenomenon has not been reported in non-*Rhizobium* PGPR so far. A main reason for aggregation of FZB42 cells on root hairs may be due to abundant exudates secreted on these regions, as shown in Figure 6 (Panel C). According to the microarray result, root exudates could trigger a vast array of biological responses of FZB42; on the other hand, bacterial activities can affect root developments [Lopez-Bucio *et al*. 2007]. Therefore, it is highly likely that root hairs play an important role in plant-microbe interactions.

Despite the similarity in terms of favoring root hairs, colonization patterns of FZB42 on the tips of primary roots of *Arabidopsis* and maize varied significantly. While the tips of *Arabidopsis* were strongly favored by FBZ42, few bacterial cells could be observed on those of maize seedlings. This difference may be explained in that maize roots grew too fast in the gnotobiotic system, far exceeding the spreading speed of bacteria on root surfaces [Bahme *et al*. 1987]. Nevertheless, other possible reasons can not be excluded. For example, the tip structures of the two kinds of primary roots were apparently different. While there were much exudates available from the lubricative layers around root tips of *Arabidopsis*, little sloughs, which can be utilized by FZB42 as nutrients, were observed nearby maize root tips, possibly due to the tight structure of maize root tips.

On some surfaces of *Lemna* roots FZB42 cells accumulated along the grooves between epidermis cells (Figure 9, Panel E&F). A similar phenomenon seems to occur on *Arabidopsis* as well (Figure 7, Panel F). It is unlikely that just by chance FZB42 cells favored these niches such as the concavities on maize root surfaces, the bifurcation sites between primary roots and lateral roots of maize and *Arabidopsis*, the grooves between neighbored epidermis cells on *Lemna* root surfaces, and the indented intercellular spaces on ventral surfaces of *Lemna* fronds. Since the morphology of maize roots was

significantly different at the time of observation from that at the moment of inoculation, the possibility can be excluded that more bacterial cells were attached to the not-yet-formed niches upon inoculation. As to *Lemna*, the other parts of root surfaces and ventral sides of fronds should have the same opportunity to contact with FZB42. Therefore, one possible explaination for the "niche phenomenon" is that the niches provide a relatively isolated microenvironment for bacteria to accommodate, propagate, and finally transform to a favored habitat.

4.1.3 Biofilm formation on root surfaces

Root colonization by rhizosphere bacteria is linked with biofilm formation [Watnick *et al*. 1999; Bais *et al*. 2004; Ramey *et al*. 2004; Reva *et al*. 2004]. Obvious differences exist between biofilms formed by FZB42 on maize roots and those on *Lemna* (Figure 6 and Figure 9). Unlike what was observed on *Lemna*, highly structured biofilms were not detected on maize roots, although microcolonies were often seen on them (Figure 6). This difference may result from factors such as plant tissue, water availability, and nutrient richness. All these factors were different between the two systems but are known to affect biofilm formation strongly [Jones *et al*. 2003; Kinsinger *et al*. 2003; Ramey *et al*. 2004; van de Mortel *et al*. 2004].

B. amyloliquefaciens FZB42 is a potent producer of cyclic lipopeptides such as surfactin, fengycin, and bacillomycin D. Among them surfactin has been demonstrated to be an important player in the formation of a stable biofilm and in facilitating cell spreading of *B. subtilis* by reducing surface tension [Bais *et al*. 2004; Leclere *et al*. 2006]. According to unpublished results obtained by Anto Budiharjo and Joachim Vater, surfactin was detected in the extracts of *Lemna* plantlets inoculated with FZB42, but not in the extracts of the control lacking FZB42 inoculation. Meanwhile, no other lipopeptides and polyketides such as bacillaene, difficidin and macrolactin, which are normally expressed by FZB42 in Landy medium, was detected in the same extracts of the treatment. These facts imply that surfactin is involved in the biofilm formation of FZB42 on *Lemna*.

4.1.4 Colonization of FZB42 on *Lemna minor*

It is not surprising that FZB42 can colonize the roots of maize and *Arabidopsis*, since root colonization of these two kinds of plants by other PGPR like *Pseudomonas* has been

reported. However, it is quite encouraging to find that FZB42 is also able to colonize *Lemna*, the smallest flower plant in the world, which is suitable for miniaturized micro-titer plate experiment. In the previous work FZB42 was demonstrated to be able to promote *Lemna* growth [Idris *et al*. 2007]. The two facts suggested that *Lemna minor* is a potential tool for investigations of plant-microbe interactions, especially taking into consideration other advantages it has: a smaller size, a simpler structure, a rapid propagation speed and the easiness to be inoculated, maintained and observed owing to the aquatic environments it requires. Furthermore, *L. minor* contains rich chlorophyll throughout fronds and roots and therefore emits red autofluorescence upon UV-excitation, which has greatly facilitated the monitoring of GFP-labeled FZB42 in this study.

Observing the colonization development of FZB42 over time on different plants and comparing its colonization patterns among them would deepen our insights into the interactions between Gram-positive PGPR and Plants. However, due to the limitation of gnotobiotic system, conducting an investigation in a more complicated soil environment, or natural water in terms of *Lemna,* may be considered in future.

4.2 Transcriptomic analysis of *B. amyloliquefaciens* FZB42 in response to maize root exudates

4.2.1 Components of the maize root exudates

Since organic acids, amino acids, mono- and oligosaccharides are thought to be the major constituents of plant root exudates, a total of 37 components of these kinds were assayed for their amount in the maize root exudates used in this work. The result showed that organic acids, amino acids and sugars accounted for only 7.7%, 3.6% and 2.0% of dry weight of the crude exudates. Moreover, nearly one fifth in dry weight of the crude exudates was insoluble and the dissolved exudates exhibited some sediment again after freezing-melting, which had to be spin-down before HPLC assay. Taking these facts together, it can be inferred that the detected components are just a small portion of the crude exudates collected. A significant part, which was not shown in Figure 10, of the exudates may at least include components such as sloughed root epidermic tissues, mucilage of high molecular weight, and some VOCs of low molecular weight.

4.2.2 OD1.0 vs. OD3.0

In contrast to a few genes at the exponential phase (OD1.0), hundreds of genes at the transient phase (OD3.0) were differentially expressed in presence of root exudate. Such a difference is not unexpected. While most transcriptions during exponential phase is typically initiated by RNAP holoenzyme carrying the housekeeping σ^A, at late exponential phase bacteria have to recruit their regulation machinery to adapt to the changing environment. Rhizobacteria may use a similar adaptive mechanism within response to the dynamic microenvironment in a rhizosphere. The kind of relevance is supported by the finding that many virulence-associated factors appear to influence colonization, persistence and spreading mechanisms of human pathogen *Streptococcus pyogenes*, in a growth phase-related fashion [Kreikemeyer *et al*. 2003; Beyer-Sehlmeyer *et al*. 2005; Chaussee *et al*. 2008].

4.2.3 NE vs. RE

Conventionally, root exudates are collected from the plants grown in a gnotobiotic system starting from surface-sterilized seeds. Since a two-way signalling is involved in the plant-microbe interaction, rhizosphere microflora will influence the compositions of root exudates by affecting root cell leakage, cell metabolism, and plant nutrition status [Yang *et al*. 2000]. Wang *et al*. reported that the colonization of *P. fluorescence* triggers a series of responses of *Arabidopsis* including an up-regulation of genes involved in metabolism, signal transduction, stress response, and putative auxin-regulated genes [Wang *et al*. 2005]. Moreover, *P. aeruginosa* also produces *N*-acyl homoserine lactone (AHL) signaling compounds that induce changes in the exudation from plants [Mathesius *et al*. 2003]. Therefore, the root exudates elicited under the condition of plant-microbe interaction should be different from the ones collected from a gnotobiotic system; and the specific exudate compounds induced or repressed by microbe in the former condition will, in turn, affect the microbe associated with plant roots. Taking this into account, an “interaction exudates (IE)” were collected from maize roots which were inoculated with FZB42 as performed in colonization experiments. The transcriptomic response of FZB42 to the “IE” was compared with that to the conventional “root exudates (RE)”.

The result showed that there was no significant difference ($q \leq 0.01$ and $FCH \geq 1.5$) between the effect of IE and RE at OD1.0, while four genes were differentially expressed

at OD3.0 as highlighted in Table 23. When a less stringent condition (q≤0.05 and FCH≥1.5) was applied, nine genes were differentially expressed (Table 23). The number of the genes obtained is much less than we expected. It is postulated that many subtle differences in composition between the two exudates were not significant enough to be revealed by the method of two-color microarray.

Table 23: The differentially expressed genes of FZB42 responding to IE compared with that to RE

Gene	*Product*	*q value*	*FCH* *wt+IE<>wt+RE*
Ggt	gamma-glutamyltranspeptidase Ggt	0.00	2.2
RBAM00438	hypothetical protein RBAM00438	0.00	1.5
nprE	bacillolysin precursor NprE	0.01	1.5
clpP	ATP-dependent Clp protease proteolytic subunit ClpP	0.00	1.5
ywcE	hypothetical protein YwcE	0.02	1.5
ydjO	hypothetical protein YdjO	0.02	1.7
RBAM03284	ribonuclease precursor (Barnase) RBAM03284	0.02	1.5
bglS	endo-beta-1,3-1,4 glucanase BglS	0.05	1.6
RBAM00226	hypothetical protein RBAM00226	0.04	-1.6

Remarks: Abbreviations used here represents, respectively: FCH: fold change; wt: FZB42 wild type; IE: "interaction exudates"; RE: root exudates; +: in the presence of root exudates or soil extract. The genes heighted in yellow were those with a q value of ≤0.01.

4.2.4 Limitations of the investigation system

Although transcriptomic profiling has successfully been done, it is important to consider limitations of the system used in this work.

One limitation of this system is that some effects of the exudates may have been overwhelmed or inhibited by components in 1CS medium and therefore did not revealed in the results as genes with altered expression. On the other hand, using 0.25 mg exudates per ml medium, some components in the exudates may be diluted to a level at which they no longer show detectable effect on bacterial gene expression.

A second limitation is that the exudates used in this work were a pool of exudates collected within seven days after maize seedlings were transferred into tubes. It is known that the compositions of root exudates are affected by plant age [Haichar *et al*. 2008]. Therefore, further improvements of the approach may include using exudates collected in several

successive but narrowed time courses. Profiling the effects of these exudates respectively may reflect the development of a rhizobactium's colonization on plant roots as the plants grow.

The third notable point is associated with data processing. In the early years of microarray application, fold change was a widely-used cutoff to filter the genes which were regarded to be differentially expressed. However, setting a cutoff of fold change is completely an arbitrary step compared with statistical analysis. In this work the expression of nearly 800 genes were significantly altered, with a fold change of ≤1.5, in response to root exudates according to statistical analysis (q≤0.01). Excluding these genes as differentially expressed ones in light of the arbitrary condition "FCH≥1.5" will greatly underestimate the number of genes which were influenced by root exudates. However, as most biologists do out of practical reasons, I also emphasized on analyzing those genes that were not only differently expressed from the respective of statistics but also have a relative high fold change (FCH≥1.5 or FCH≥2.0 in this work).

4.3 Alternative sigma factors, AbrB, DegU and the response of FZB42 to root exudates

In this work seven protein factors affecting bacterial transcription were studied to determine which genes are regulated by them and if they are involved in the transcriptional response of FZB42 to root exudates. The same filter condition (q≤0.01 and FCH≥2.0) was applied to the analysis for all factors except SigD and DegU, for which a modified condition was used because more biological replicates were used in the two cases. It is shown that the numbers of genes regulated by the various factors varied a lot. For example, while SigB was indentified to regulate no more than 30 genes and to be involved in only 2 genes' response to root exudates, AbrB was shown to effect the expression of more than 1000 genes and to be involved in 149 genes' response to root exudates. This great difference could mainly result from the distinctions between the intrinsic properties of the regulators. For instance, AbrB is known to be a most important transition-state regulator orchestrating the expressions of a vast array of genes; however, as a general but in many cases not essential stress sigma factor, the functions of SigB still remain somewhat elusive. Besides, the regulatory overlap among the ECF sigma factors [Mascher *et al*. 2007] may also affect the significance of a single ECF sigma factor on gene expression of FZB42. Mascher *et al* reported that several ECF σ factor genes (*sigM, sigV, sigW, sigX, Ylac*) in *B.*

subtilis have promoters sharing much similarity and display a significant regulatory overlap so that the null mutation of an ECF σ factor gene shows no dramatic phenotypes, probably because one of the ECF sigma factors could be functionally replaced by other redundant ones. Finally, external factors like system errors may also contribute to this difference, since all experiments were performed independent of the others.

DNA microarray provides a high throughput method to identify systematically the genes regulated by alternative sigma factors or other regulators [Ogura *et al*. 2001; Asai *et al*. 2003; Serizawa *et al*. 2004; Stephan *et al*. 2005]. On one hand, it is nearly inevitable that some false positive results will be produced by this method; one the other hand, some truly positive genes may be omitted from the final results, owing to the factors like stringency setting. For example, the genes shown in Table 24 reside in two operons responsible for the synthesis of the dipeptide bacilysin and another new antibiotic, respectively. The production of the two antibiotics has recently been confirmed in our lab to be positively regulated by DegU, but they would be excluded, except RBAM_029240, from the result obtained if applying a condition of FCH≥2.0. As to the genes left in the final lists, some of them have previously been reported but most of them still need to be further confirmed experimentally.

Table 24: The genes identified to be positively regulated by DegU

Gene	degU-RE<> wt-RE		degU +RE<> wt +RE		Product
	q value	FCH	q value	FCH	
bacA	0.00	-1.3	0.00	-1.6	bacilysin synthetase A (BacA)
bacB	0.00	-1.7	0.00	-1.5	bacilysin synthetase B (BacB)
bacC	0.00	-1.6	0.00	-1.4	bacilysin synthetase C (BacC)
bacD	0.00	-1.2	0.00	-1.5	bacilysin synthetase, amino acid ligase subunit (BacD)
bacE	0.00	-1.3	0.00	-1.6	anticapsin/bacilysin excretion protein (BacE)
RBAM_029230	0.00	-1.5	0.00	-1.8	hypothetical protein RBAM_029230
RBAM_029240	0.00	-1.6	0.00	-2.4	hypothetical protein RBAM_029240

Remarks: Abbreviations used here represents, respectively: wt: FZB42 wild type; degU: FZB42 Δ*degU*; RE: root exudates; +: in the presence of root exudates or soil extract; -: without root exudates or soil extract.

It is noteworthy that some genes were identified to be regulated by more than one regulator. For instances, *hag* was positively regulated by SigD while negatively controlled by DegU. According to the known knowledge, SigD is a component of the holoenzyme transcribing *hag*, while phosphorylated DegU represses the transcription of SigD by

binding, at least *in vitro*, to the regulatory region of the *fla-che* operon [Amati *et al*. 2004]. Accordingly, the complexity of interactions between global transcriptional regulators and sigma factors must be taken into consideration in data analyzing.

4.4 sRNAs involved in the response of FZB42 to root exudates

Six sRNAs in *B. amyloliquefaciens* FZB42 were identified and the expression of some of them in response to root exudates was confirmed. It is reported for the first time that sRNAs are involved in plant-microbe interaction, although more work still needs to be done. The interesting work in the future may include, for example, determining which composition(s) in the exudates directly resulted in the altered expression of the sRNAs; determining the complete sequence of the sRNAs by primer extension or 5'-RACE; and figuring out the target genes regulated by the sRNAs.

Regarding the intriguing sRNA Igr3927, its complete sequence could be determined *in silico* (Figure 21) according to its length shown in northern blot and the multiple alignment result. With this sequence, the target mRNAs of Igr3927 were predicted by using an online program *TargetRNA* [Tjaden *et al*. 2006]. Five possible target genes of Igr3927 were obtained (Figure 22) and their responses to root exudates were shown in Table 25.

Rank	Gene	Synonym	Score	Pvalue
1	ywtG	RBAM_032960	-60	0.00220349
2	ydbR	RBAM_004920	-58	0.00330777
3	gmk	RBAM_015510	-55	0.00608034
4	ansA	RBAM_021710	-54	0.00744666
5	yvrH	RBAM_018130	-53	0.00911861

mRNA (ansA):

...GCUUAACACAUCUUGAGAAAUGGAUGAAGAGGUGCAGGGUAUGAAAAAAUUAUUGCUGUUAACAACCGGAGGAACAA...

Figure 22: The predicted target genes of sRNA Igr3927.

The region of *ansA* mRNA highlighted in yellow suggests the possible base paring sequence with Igr3927, while the gray region belongs to the coding sequence of *ansA*.

Among the five genes, only *ansA* encoding asparaginase was differentially expressed responding to the root exudates (Table 25). Asparagine is one component found in the exudates used. Therefore, I came up with the following assumption: The transcription of *ansA* was repressed by Igr3927 under conditions of asparagines starvation, probably via the base pairing of Igr3927 with the 5'-UTR of *ansA* mRNA (the region highlighted in

yellow in Figure 22). When the root exudates were applied, the expression of Igr3927 was inhibited by the asparagine present in root exudates (Figure 20), and its repression on *ansA* was thus relieved, resulting in an induced expression of AnsA (Table 25), which catalyzes the hydrolysis of asparagine to aspartic acid. If this assumption can be confirmed, it will broaden our insights into the molecular mechanisms of how bacteria response to signals from plants.

Table 25: The transcriptional response of the target genes of Igr3027 to root exudates

gene	q value	FCH	product
ywtG	0.44	-1.1	putative transport protein YwtG
ydbR	0.20	-1.1	putative ATP-dependent RNA helicase YdbR
gmk	0.03	-1.2	putative guanylate kinase Gmk
ansA	0.00	1.6	L-asparaginase AnsA
yvrH	0.09	-1.1	two-component system response regulator YvrH

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6 Appendix

Appendix Table 1: The genes of FZB42 with known function which were significantly differentially expressed in response to maize root exudates

Gene	*Product*	*Fuctional catagory*	*FCH*	*Transcriptional factors involved*
		1_cell envelope and cellular processes		
divIC	cell-division initiation protein DivIC	1.7_ Cell division	1.7	
ftsH	cell division protein and general stress protein(class III heat-shock protein) FtsH	1.7_ Cell division	1.5	AbrB
ftsL	cell-division protein FtsL	1.7_ Cell division	1.7	AbrB
ftsZ	cell-division initiation protein FtsZ	1.7_ Cell division	1.7	
minC	cell-division inhibitor (septum placement) MinC	1.7_ Cell division	1.6	
ywkC	cell division protein: attaches the chromosome to the cell pole	1.7_ Cell division	-1.5	
pbpF	penicillin-binding protein 2C PbpF	1.1_ Cell wall	1.5	
murB	UDP-N-acetylenolpyruvoylglucosamine reductase MurB	1.1_ Cell wall	1.6	
tuaB	teichuronic acid biosynthesis protein TuaB	1.1_ Cell wall	-1.5	
ymfM	required for cell shape determination	1.1_ Cell wall	1.5	
yoeB	inhibits in vitro activity of cell wall endopeptidases LytE and LytF, inhibits cell separation	1.1_ Cell wall	1.6	AbrB
yjlD	NADH dehydrogenase-like protein YjlD	1.4_ Membrane bioenergetics	1.6	AbrB
resA	thiol-disulfide oxidoreductase ResA	1.4_ Membrane bioenergetics	1.7	DegU, SigD
atpC	ATP synthase (subunit epsilon) AtpC	1.4_ Membrane bioenergetics	1.6	AbrB, SigX
atpH	ATP synthase (subunit delta) AtpH	1.4_ Membrane bioenergetics	1.7	AbrB
atpF	ATP synthase (subunit B) AtpF	1.4_ Membrane bioenergetics	1.5	AbrB
qoxB	quinol oxidase polypeptide I QoxB	1.4_ Membrane bioenergetics	1.6	AbrB
qoxA	quinol oxidase subunit II precursor QoxA	1.4_ Membrane bioenergetics	1.6	AbrB
fliM	flagellar motor switch protein FliM	1.5_ Mobility and chemotaxis	2.0	SigX
fliP	flagellar biosynthetic protein FliP	1.5_ Mobility and chemotaxis	1.7	AbrB
cheC	chemotaxis protein CheC	1.5_ Mobility and chemotaxis	1.7	AbrB
cheD	chemotaxis protein CheD	1.5_ Mobility and chemotaxis	-1.5	
hag	flagellin proteinHag	1.5_ Mobility and chemotaxis	3.6	AbrB, DegU, SigD, SigX
flgM	negative regulator of flagellin synthesis (Anti-sigma-D factor) FlgM	1.5_ Mobility and chemotaxis	1.7	SigX
luxS	s-ribosylhomocysteine lyase LuxS	1.3_ Sensors (signal transduction)	1.7	AbrB, SigD
ymcA	antagonist of biofilm repression by SinR, regulation of biofilm formation	1.3_ Sensors (signal transduction)	2.5	AbrB, SigD
secE	preprotein translocase subunit SecE	1.6_ Protein secretion	1.7	AbrB, SigD
secY	preprotein translocase subunit SecY	1.6_ Protein secretion	2.0	AbrB, SigX
tatAy	sec-independent protein translocase protein TatAy	1.6_ Protein secretion	1.6	AbrB
tatCy	sec-independent protein translocase protein TatCy	1.6_ Protein secretion	1.6	
lytA	membrane bound lipoprotein LytA	1.6_ Protein secretion	1.5	AbrB

rapA	response regulator aspartate phosphatase RapA	1.8_ Sporulation	1.7	DegU, SigD
ypeB	sporulation protein YpeB	1.8_ Sporulation	1.5	
sda	sporulation inhibitor Sda	1.8_ Sporulation	1.7	AbrB, SigD
spoIIB	endospore development protein SpoIIB	1.8_ Sporulation	-1.7	SigD, SigM
sspI	small acid-soluble spore protein SspI	1.8_ Sporulation	-1.7	
cotG	spore coat protein G (CotG)	1.8_ Sporulation	1.7	
yabP	required for sporulation at a late stage	1.8_ Sporulation	2.1	SigX
comS	competence protein S ComS	1.1_ Transformation/competence	1.7	AbrB, DegU, SigM
med	transcriptional activator protein med precursor Med	1.1_ Transformation/competence	-1.6	SigM, SigV
gutA	probable glucitol transport protein GutA	1.2_ Transport/binding proteins and lipoproteins	2.8	AbrB
citM	magnesium citrate secondary transporter CitM	1.2_ Transport/binding proteins and lipoproteins	2.4	
glvC	phosphotransferase system (PTS) maltose-specificenzyme IICB component GlvC	1.2_ Transport/binding proteins and lipoproteins	2.5	
appF	oligopeptide transport ATP-binding protein AppF	1.2_ Transport/binding proteins and lipoproteins	1.5	
oppA	oligopeptide ABC transporter (binding protein) OppA	1.2_ Transport/binding proteins and lipoproteins	1.5	
oppD	oligopeptide ABC transporter (ATP-binding protein) OppD	1.2_ Transport/binding proteins and lipoproteins	1.5	AbrB, DegU
oppF	oligopeptide ABC transporter (ATP-binding protein) OppF	1.2_ Transport/binding proteins and lipoproteins	1.6	DegU
cysP	sulfate permease CysP	1.2_ Transport/binding proteins and lipoproteins	1.8	
ebrB	multidrug resistance protein EbrB	1.2_ Transport/binding proteins and lipoproteins	1.8	SigD
araQ	L-arabinose transport system permease protein AraQ	1.2_ Transport/binding proteins and lipoproteins	1.9	AbrB
araP	L-arabinose transport system permease protein AraP	1.2_ Transport/binding proteins and lipoproteins	2.4	
araN	probable arabinose-binding protein precursor AraN	1.2_ Transport/binding proteins and lipoproteins	2.2	
amyC	maltose transport protein AmyC	1.2_ Transport/binding proteins and lipoproteins	1.8	SigD
mscL	Large conductance mechanosensitive channel protein MscL	1.2_ Transport/binding proteins and lipoproteins	1.8	AbrB
licA	phosphotransferase system (PTS) lichenan specific enzyme IIA component LicA	1.2_ Transport/binding proteins and lipoproteins	1.5	
iolF	inositol transport protein IolF	1.2_ Transport/binding proteins and lipoproteins	2.1	
rocE	amino acid permease RocE	1.2_ Transport/binding proteins and lipoproteins	4.0	AbrB
ykoE	thiamine ABC transporter (membrane protein), thiamine uptake	1.2_ Transport/binding proteins and lipoproteins	-1.5	
		2_intermediary metabolism		
acoL	acetoin dehydrogenase E3 component (dihydrolipoamide dehydrogenase) AcoL	2.1_ Metabolism of carbohydrates and related molecules	1.5	AbrB
ald	alanine dehydrogenase Ald	2.2_ Metabolism of amino acids and related molecules	-2.4	
ansA	L-asparaginase AnsA	2.2_ Metabolism of amino acids and related molecules	1.6	AbrB
araB	L-ribulokinase AraB	2.1_ Metabolism of carbohydrates and related molecules	1.7	AbrB
araD	L-ribulose-5-phosphate 4-epimerase AraD	2.1_ Metabolism of carbohydrates and related molecules	1.8	

araL	arabinose operon protein L (AraL)	2.1_ Metabolism of carbohydrates and related molecules	2.6	AbrB
araM	arabinose operon protein M (AraM)	2.1_ Metabolism of carbohydrates and related molecules	2.3	AbrB
citB	aconitate hydratase CitB	2.1_ Metabolism of carbohydrates and related molecules	1.7	AbrB
citZ	citrate synthase II CitZ	2.1_ Metabolism of carbohydrates and related molecules	2.3	AbrB
galE1	UDP-glucose 4-epimerase GalE1	2.1_ Metabolism of carbohydrates and related molecules	1.6	
galK1	galactokinase GalK1	2.1_ Metabolism of carbohydrates and related molecules	5.3	
galT1	galactose-1-phosphate uridyltransferase GalT1	2.1_ Metabolism of carbohydrates and related molecules	4.2	AbrB
gapB	glyceraldehyde-3-phosphate dehydrogenase GapB	2.1_ Metabolism of carbohydrates and related molecules	1.6	AbrB
gcvPB	glycine decarboxylase (subunit 2) (glycine cleavage system protein P) GcvPB	2.2_ Metabolism of amino acids and related molecules	1.6	AbrB
gcvT	aminomethyltransferase (glycine cleavage system protein T) GcvT	2.2_ Metabolism of amino acids and related molecules	1.8	AbrB
glpK	glycerol kinase (ATP:glycerol 3-phosphotransferase) (Glycerokinase) GlpK	2.1_ Metabolism of carbohydrates and related molecules	1.5	AbrB
glvA	maltose-6'-phosphate glucosid GlvA	2.1_ Metabolism of carbohydrates and related molecules	5.2	AbrB, DegU
gudB	NAD-specific glutamate dehydrogenase GudB	2.2_ Metabolism of amino acids and related molecules	1.5	AbrB, DegU
iolA	methylmalonate-semialdehyde dehydrogenase IolA	2.2_ Metabolism of amino acids and related molecules	2.7	AbrB
iolB	inositol utilization protein B (IolB)	2.1_ Metabolism of carbohydrates and related molecules	2.7	AbrB
iolC	inositol utilization protein C (IolC)	2.1_ Metabolism of carbohydrates and related molecules	4.2	
iolD	inositol utilization protein D (IolD)	2.1_ Metabolism of carbohydrates and related molecules	4.2	
iolE	inositol utilization protein E (IolE)	2.1_ Metabolism of carbohydrates and related molecules	2.8	
iolG	myo-inositol 2-dehydrogenase IolG	2.1_ Metabolism of carbohydrates and related molecules	2.5	
iolI	inositol utilization protein I (IolI)	2.1_ Metabolism of carbohydrates and related molecules	2.0	
iolS	inositol utilization protein S (IolS)	2.1_ Metabolism of carbohydrates and related molecules	1.7	AbrB
kbl	2-amino-3-ketobutyrate coenzyme A ligase Kbl...	2.2_ Metabolism of amino acids and related molecules	2.2	
lacE	phosphotransferase system (PTS) lichenan-specific enzyme IIC component LacE	2.1_ Metabolism of carbohydrates and related molecules	1.6	AbrB
lacF	phosphotransferase system cellobiose-specific component LacF	2.1_ Metabolism of carbohydrates and related molecules	1.8	AbrB
licH	6-phospho-beta-glucosidase LicH	2.1_ Metabolism of carbohydrates and related molecules	1.6	
mdh	malate dehydrogenase Mdh	2.1_ Metabolism of carbohydrates and related molecules	1.9	AbrB
odhB	dihydrolipoyllysine-residue succinyltransferase component of 2-oxoglutarate dehydrogenase complex OdhB	2.1_ Metabolism of carbohydrates and related molecules	2.0	
pdhC	pyruvate dehydrogenase (dihydrolipoamide acetyltransferase E2 subunit) PdhC	2.1_ Metabolism of carbohydrates and related molecules	1.5	
pgi	glucose-6-phosphate isomerase Pgi	2.1_ Metabolism of carbohydrates and related molecules	1.5	AbrB
pgk	phosphoglycerate kinase Pgk	2.1_ Metabolism of carbohydrates and related molecules	2.4	
pgm2	phosphoglyceromutase Pgm2...	2.1_ Metabolism of carbohydrates	1.8	AbrB

		and related molecules		
proA	gamma-glutamyl phosphate reductase ProA	2.2_ Metabolism of amino acids and related molecules	-1.6	
rocD	ornithine aminotransferase RocD	2.2_ Metabolism of amino acids and related molecules	6.5	AbrB
rocF	arginase RocF	2.2_ Metabolism of amino acids and related molecules	5.4	
rpe	ribulose-5-phosphate 3-epimerase Rpe	2.1_ Metabolism of carbohydrates and related molecules	1.5	AbrB
sdhB	succinate dehydrogenase (iron-sulfur protein) SdhB	2.1_ Metabolism of carbohydrates and related molecules	1.5	
sucC	succinyl-CoA synthetase (beta subunit) SucC	2.1_ Metabolism of carbohydrates and related molecules	1.9	AbrB, SigD
sucD	succinyl-CoA synthetase (alpha subunit) SucD	2.1_ Metabolism of carbohydrates and related molecules	1.7	AbrB, SigX
tdh	L-threonine 3-dehydrogenase Tdh	2.2_ Metabolism of amino acids and related molecules	3.2	
thrB	homoserine kinase ThrB	2.2_ Metabolism of amino acids and related molecules	-1.5	
ydjE	fructokinase homolog YdjE	2.1_ Metabolism of carbohydrates and related molecules	1.6	
pabC	aminodeoxychorismate lyase PabC	2.5_ Metabolism of coenzymes and prosthetic groups	1.7	
hepT	heptaprenyl diphosphate synthase component II HepT	2.5_ Metabolism of coenzymes and prosthetic groups	2.0	AbrB
folC	folyl-polyglutamate synthetase FolC	2.5_ Metabolism of coenzymes and prosthetic groups	1.7	
ywkE	hemK protein homolog YwkE	2.5_ Metabolism of coenzymes and prosthetic groups	1.6	AbrB
scoB	succinyl CoA:3-oxoacid CoA-transferase (subunit B) ScoB	2.4_ Metabolism of lipids	1.6	DegU, SigD
yngG	hydroxymethylglutaryl-CoA lyase homolog YngG	2.4_ Metabolism of lipids	-1.5	
bkdB	branched-chain alpha-keto acid dehydrogenase E2 subunit (lipoamide acyltransferase) BkdB	2.4_ Metabolism of lipids	1.9	
bkdAA	branched-chain alpha-keto acid dehydrogenase E1 subunit (2-oxoisovalerate dehydrogenase alpha) bBkdAA	2.4_ Metabolism of lipids	1.7	AbrB
bcd	leucine dehydrogenase Bcd	2.4_ Metabolism of lipids	1.8	AbrB
nin	inhibitor of the DNA degrading activity of NucA (competence) Nin	2.3_ Metabolism of nucleotides and nucleic acids	1.5	AbrB
pyrF	orotidine 5'-phosphate decarboxylase PyrF	2.3_ Metabolism of nucleotides and nucleic acids	-1.6	DegU
pyrH	uridylate kinase PyrH	2.3_ Metabolism of nucleotides and nucleic acids	1.5	AbrB
cdd	cytidine deaminase Cdd	2.3_ Metabolism of nucleotides and nucleic acids	1.7	AbrB, DegU, SigD
		4_other functions		
ykrL	protease htpx homolog YkrL	4.1_ Adaptation to atypical conditions	1.5	
degR	regulatory protein DegR	4.1_ Adaptation to atypical conditions	1.5	DegU
grpE	heat-shock protein GrpE	4.1_ Adaptation to atypical conditions	1.5	
ytxG	general stress protein	4.1_ Adaptation to atypical conditions	1.5	AbrB, SigD
yqjL	general stress protein, putative hydrolase involved in oxidative stress resistance	4.1_ Adaptation to atypical conditions	1.5	AbrB
yqeZ	seine protease, resistence protein (against sublancin)	4.1_ Adaptation to atypical conditions	2.0	DegU
yceD	general stress protein, similar to tellurium resistance protein	4.2_ Detoxification	1.7	
yceE	general stress protein, similar to tellurium resistance protein	4.2_ Detoxification	1.8	AbrB

yceF	general stress protein, similar to tellurium resistance protein	4.2_ Detoxification	1.7	AbrB
yfhL	general stress protein, resistence protein (against toxic peptide SdpC)	4.2_ Detoxification	1.5	
ctaG	formation of functional cytochrome C-oxidase (caa3)	4.6_ Miscellaneous	1.5	
era	GTP-binding protein Era	4.6_ Miscellaneous	2.2	AbrB, DegU
yurV	iron-sulfur cofactor synthesis protein nifU homolog YurV	4.6_ Miscellaneous	1.7	AbrB
xhlA	phage-like element PBSX protein XhlA	4.4_ Phage-related functions	1.6	AbrB
baeE	malonyl-CoA-[acyl-carrier protein] transacylase BaeE	4.3_ Antibiotic production	1.6	AbrB
baeI	enoyl-CoA-hydratase BaeI	4.3_ Antibiotic production	2.2	
baeL	polyketide synthase BaeL	4.3_ Antibiotic production	1.9	
baeN	hybrid NRPS/PKS BaeN	4.3_ Antibiotic production	1.5	AbrB
baeR	polyketide synthase BaeR	4.3_ Antibiotic production	2.3	AbrB
difJ	modular polyketide synthase of type I DifJ	4.3_ Antibiotic production	2.0	AbrB, SigD
difI	modular polyketide synthase of type I DifI	4.3_ Antibiotic production	1.7	AbrB, SigD
difG	modular polyketide synthase of type I DifG	4.3_ Antibiotic production	2.0	AbrB
difF	modular polyketide synthase of type I DifF	4.3_ Antibiotic production	2.4	SigD
mlnH	polyketide synthase of type I MlnH	4.3_ Antibiotic production	1.5	AbrB
fenE	fengycin synthetase FenE	4.3_ Antibiotic production	1.5	DegU, SigD
srfAD	surfactin synthetase D SrfAD	4.3_ Antibiotic production	1.9	AbrB, DegU, SigD, SigM
srfAC	surfactin synthetase C SrfAC	4.3_ Antibiotic production	1.7	AbrB, DegU
		3_information pathways		
recA	multifunctional SOS repair regulator RecA	3.3_ DNA recombination	1.6	AbrB
priA	primosomal protein N' PriA	3.1_ DNA replication	1.5	
ssb	single-strand DNA-binding protein (Helix-destabilizing protein) Ssb	3.1_ DNA replication	1.6	AbrB, SigX
yneE	sporulation protein, inhibits DNA replication, control of chromosome copy number	3.1_ DNA replication	-1.5	
map	methionine aminopeptidase Map	3.8_ Protein modification	3.1	SigX
prpC	protein phosphatase PrpC	3.8_ Protein modification	1.7	
alaS	alanyl-tRNA synthetase AlaS	3.7_ Protein synthesis	-1.5	
fusA	elongation factor G FusA	3.7_ Protein synthesis	2.2	AbrB, SigX
tufA	elongation factor Tu TufA	3.7_ Protein synthesis	1.5	AbrB, SigX
lepA	GTP-binding protein LepA	3.7_ Protein synthesis	1.5	
infA	translation initiation factor IF-I InfA	3.7_ Protein synthesis	2.0	SigX
infB	initiation factor (IF-2) InfB	3.7_ Protein synthesis	1.6	
infC	initiation factor IF-3 InfC	3.7_ Protein synthesis	1.8	
rplA	ribosomal protein L1 (BL1) RplA	3.7_ Protein synthesis	1.7	AbrB, SigX
rplJ	ribosomal proteinL10 (BL5) RplJ	3.7_ Protein synthesis	2.0	AbrB, SigX
rplD	ribosomal protein L4 RplD	3.7_ Protein synthesis	1.8	AbrB, SigX
rpsM	ribosomal protein S13 RpsM	3.7_ Protein synthesis	1.6	SigD, SigX
rpsK	ribosomal protein S11 (BS11) RpsK	3.7_ Protein synthesis	1.6	AbrB, SigX
rplM	ribosomal protein L13 RplM	3.7_ Protein synthesis	1.8	AbrB, SigD
rpsI	ribosomal protein S9 RpsI	3.7_ Protein synthesis	1.7	AbrB
rpsO	ribosomal protein S15 (BS18) RpsO	3.7_ Protein synthesis	1.6	AbrB
rpmGA	50S ribosomal protein L33 type I RpmGA	3.7_ Protein synthesis	1.7	AbrB, DegU

rpsU	ribosomal protein S21 RpsU	3.7_ Protein synthesis	3.1	AbrB, SigD
rpmA	50S ribosomal protein L27 (BL30) (BL24) RpmA	3.7_ Protein synthesis	1.6	AbrB
rplU	50S ribosomal protein L21 (BL20) RplU	3.7_ Protein synthesis	2.0	AbrB
rpsR	ribosomal protein S18 RpsR	3.7_ Protein synthesis	2.1	AbrB, SigD, SigX
trmU	tRNA (5-methylaminomethyl-2-thiouridylate) methyltransferase TrmU	3.6_ RNA modification	1.5	AbrB
ydcE	RNase EndoA, MazF family toxin, cleaves cellular mRNAs at specific, but frequently occuring sites	3.5_ RNA synthesis	1.5	AbrB
yjbH	adaptor protein for ClpX-ClpP-catalyzed Spx degradation	3.5_ RNA synthesis	1.5	
ykqC	RNase J1, RNA processing, subject to Clp-dependent proteolysis upon glucose starvation	3.5_ RNA synthesis	1.6	AbrB
ymdA	RNase Y, 5' end sensitive endoribonuclease, involved in the degradation/processing of mRNA	3.5_ RNA synthesis	1.6	
rpoC	RNA polymerase (beta subunit) RpoC	3.5_ RNA synthesis	1.9	AbrB, SigX
rpoA	RNA polymerase (alpha subunit) RpoA	3.5_ RNA synthesis	2.0	AbrB, SigX
sigW	rNA polymerase ECF-type sigma factor SigW	3.5_ RNA synthesis	2.4	DegU, SigD
yjbD	Transcriptional regulator Spx, involved in regulation of many genes.	3.5_ RNA synthesis	1.5	AbrB, SigX
glvR	HTH-type transcriptional regulator GlvR	3.5_ RNA synthesis	4.4	AbrB, SigB
perR	peroxide operon regulator PerR	3.5_ RNA synthesis	2.2	
glpP	glycerol uptake operon antiterminator regulatoryprotein GlpP	3.5_ RNA synthesis	1.8	AbrB
hpr	protease production regulatory protein Hpr	3.5_ RNA synthesis	1.5	
fapR	transcription factor (Fatty acid and phospholipid biosynthesis regulator) FapR	3.5_ RNA synthesis	1.5	AbrB
glnR	glutamine synthetase transcription repressor GlnR	3.5_ RNA synthesis	1.8	
hrcA	heat-inducible transcription repressor HrcA	3.5_ RNA synthesis	1.9	SigM
phoP	alkaline phosphatase synthesis transcriptional regulatory protein PhoP	3.5_ RNA synthesis	1.9	AbrB
spoIIID	stage III sporulation protein D (SpoIIID)	3.5_ RNA synthesis	-1.5	AbrB, SigM
yqzJ	ribosome-nascent chain sensor of membrane protein biogenesis	3.5_ RNA synthesis	1.5	

Appendix Table 2: The genes of FZB42 with putative function or encoding hypothetical protein which were significantly differentially expressed in response to maize root exudates

Gene	***Product***	***Fuctional catagory***	***FCH***	***Transcriptional factors involved***
		1_cell envelope and cellular processes		
ykqB	conserved hypothetical protein YkqB	1.2_ Transport/binding proteins and lipoproteins	1.6	AbrB
yqeW	conserved hypothetical protein YqeW	1.2_ Transport/binding proteins and lipoproteins	-1.5	SigM
yyaJ	conserved hypothetical protein YyaJ	1.2_ Transport/binding proteins and lipoproteins	-1.6	
ytrE	hypothetical ABC transporter ATP-binding proteinYtrE	1.2_ Transport/binding proteins and lipoproteins	-1.5	
yufN	hypothetical lipoprotein YufN	1.2_ Transport/binding proteins and lipoproteins	1.7	AbrB, DegU

RBAM00714	putative ABC transporter (ATP-binding protein) RBAM00714	1.2_ Transport/binding proteins and lipoproteins	-1.5	AbrB
RBAM03581	putative ABC transporter ATP-binding protein RBAM03581	1.2_ Transport/binding proteins and lipoproteins	-1.5	
RBAM00715	putative ABC transporter permease RBAM00715	1.2_ Transport/binding proteins and lipoproteins	-1.7	AbrB, SigD
yknZ	putative ABC transporter permease YknZ	1.2_ Transport/binding proteins and lipoproteins	1.7	
ytnA	putative amino acid permease YtnA	1.2_ Transport/binding proteins and lipoproteins	1.9	DegU
ytmK	putative amino-acid ABC transporter (extracellular binding protein) YtmK	1.2_ Transport/binding proteins and lipoproteins	1.6	AbrB
cimH	putative citrate/malate transporter CimH	1.2_ Transport/binding proteins and lipoproteins	1.6	DegU
ydjK	putative sugar transporter YdjK	1.2_ Transport/binding proteins and lipoproteins	2.3	
mrsK2	putative sensor histidine kinase MrsK2	1.3_ Sensors (signal transduction)	1.5	
yacA	conserved hypothetical protein YacA	1.7_ Cell division	1.5	
		2_intermediary metabolism		
pgm1	predicted phosphatase/phosphohexomutase Pgm1	2.1_ Metabolism of carbohydrates and related molecules	2.4	AbrB, SigB
lacG	putative 6-phospho-beta-galactosidase LacG	2.1_ Metabolism of carbohydrates and related molecules	2.7	AbrB
ycsN	putative aryl-alcohol dehydrogenase YcsN	2.1_ Metabolism of carbohydrates and related molecules	1.6	AbrB, DegU
ydjL	putative dehydrogenase YdjL	2.1_ Metabolism of carbohydrates and related molecules	1.5	
epsE	putative exopolysaccharide biosynthesis protein EspE	2.1_ Metabolism of carbohydrates and related molecules	1.5	
RBAM02462	putative polysaccharide deacetylase RBAM02462	2.1_ Metabolism of carbohydrates and related molecules	-1.5	
ymfH	conserved hypothetical protein YmfH	2.2_ Metabolism of amino acids and related molecules	-1.5	
yisK	putative 5-oxo-1,2,5-tricarboxilic-3-penten aciddecarboxylase YisK	2.2_ Metabolism of amino acids and related molecules	1.6	SigD
cysC	putative adenylyl-sulfate kinase CysC	2.2_ Metabolism of amino acids and related molecules	1.5	AbrB
yurP	putative glutamine-fructose-6-phosphate transaminase YurP	2.2_ Metabolism of amino acids and related molecules	-1.9	AbrB, SigM, SigV
yurL	putative sugar kinase YurL	2.2_ Metabolism of amino acids and related molecules	-1.5	SigM
yabR	putative polyribonucleotide nucleotidyltransferase YabR	2.3_ Metabolism of nucleotides and nucleic acids	1.7	AbrB, DegU, SigD
ycsD	conserved hypothetical protein YcsD	2.4_ Metabolism of lipids	1.8	AbrB, SigM
yusL	putative 3-hydroxyacyl-CoA dehydrogenase YusL	2.4_ Metabolism of lipids	1.6	AbrB, SigD
ydbM	putative butyryl-CoA dehydrogenase YdbM	2.4_ Metabolism of lipids	1.5	AbrB
ptb	putative phosphate butyryltransferase Ptb	2.4_ Metabolism of lipids	1.7	AbrB
yvgQ	putative sulfite reductase YvgQ	2.7_Metabolism of sulfur	1.5	AbrB
		3_information pathways		
yrrC	conserved hypothetical protein YrrC	3.3_ DNA recombination	-1.5	
ydeB	conserved hypothetical protein YdeB	3.5_ RNA synthesis	2.9	AbrB, SigD
yvyD	conserved hypothetical protein YvyD	3.5_ RNA synthesis	1.8	AbrB, SigD
ybbM	predicted transmembrane transcriptional regulator (anti-sigma W factor) YbbM	3.5_ RNA synthesis	3.2	DegU, SigD
yybE	putative HTH-type transcriptional regulator YybE	3.5_ RNA synthesis	-1.7	
lacR	putative lactose phosphotransferase system repressor protein LacR	3.5_ RNA synthesis	1.5	
RBAM00542	putative transcriptional regulator (GntR family)RBAM00542	3.5_ RNA synthesis	-1.7	DegU
ybxF	conserved hypothetical protein YbxF	3.7_ Protein synthesis	2.0	SigD
yxaL	conserved hypothetical protein YxaL	3.8_ Protein modification	1.5	DegU

		4_other functions		
yceH	putative toxic anion resistance protein YceH	4.2_ Detoxification	1.7	
dfnY	hypothetical protein DifY	4.3_ Antibiotic production	1.7	
veg	conserved hypothetical proteinVeg	4.6_ Miscellaneous	2.8	AbrB, SigX

Appendix Table 3: The genes of FZB42 with unknown function which were significantly differentially expressed in response to maize root exudates.

Gene	***FCH***	***Description***	**Transcriptional factors involved**
ywcI	-4	Similar to unknown proteins from B. subtilis	DegU, SigD, SigM
RBAM01763	-2	Similar to unknown proteins from other organisms	DegU, SigD
RBAM03844	-1.8	No similarity	AbrB, SigD
yfjT	-1.8	Similar to unknown proteins from B. subtilis	AbrB, SigM, SigV
ylbK	-1.6	Similar to unknown proteins from B. subtilis	SigM
RBAM01835	-1.6	No similarity	AbrB, DegU, SigD
RBAM03862	-1.6	No similarity	
RBAM03224	-1.6	No similarity	DegU, SigD
yydA	-1.6	Similar to unknown proteins from B. subtilis	
ywqB	-1.6	Similar to unknown proteins from B. subtilis	SigV
yxxF	-1.5	Similar to unknown proteins from B. subtilis	
RBAM01125	-1.5	Similar to unknown proteins from other organisms	
RBAM01923	-1.5	No similarity	
yvqI	1.5	Similar to unknown proteins from B. subtilis	DegU, SigD
yppF	1.5	Similar to unknown proteins from B. subtilis	SigD
yqxD	1.5	Similar to unknown proteins from B. subtilis	AbrB
RBAM01955	1.5	Similar to unknown proteins from other organisms	
RBAM01886	1.5	No similarity	AbrB
ybfQ	1.5	Similar to unknown proteins from B. subtilis	
ydjI	1.5	Similar to unknown proteins from B. subtilis	
yfiT	1.5	Similar to unknown proteins from B. subtilis	SigD
yhjN	1.5	Similar to unknown proteins from B. subtilis	AbrB
yqhY	1.5	Similar to unknown proteins from B. subtilis	AbrB
ycgB	1.5	Similar to unknown proteins from B. subtilis	AbrB
ypbS	1.5	Similar to unknown proteins from B. subtilis	
yebC	1.5	Similar to unknown proteins from B. subtilis	
yjlC	1.5	Similar to unknown proteins from B. subtilis	AbrB
ypeP	1.5	Similar to unknown proteins from B. subtilis	AbrB
ybbR	1.5	Similar to unknown proteins from B. subtilis	
RBAM00685	1.5	No similarity	
yfhH	1.6	Similar to unknown proteins from B. subtilis	
yaaR	1.6	Similar to unknown proteins from B. subtilis	AbrB
RBAM01042	1.6	Similar to unknown proteins from other organisms	AbrB
ylbN	1.6	Similar to unknown proteins from B. subtilis	AbrB, SigD
ylqD	1.6	Similar to unknown proteins from B. subtilis	
ywlA	1.6	Similar to unknown proteins from B. subtilis	AbrB
RBAM02992	1.6	No similarity	AbrB
RBAM02215	1.6	Similar to unknown proteins from other organisms	
yrrK	1.6	Similar to unknown proteins from B. subtilis	
yrzL	1.6	Similar to unknown proteins from B. subtilis	

ypmA	1.6	Similar to unknown proteins from B. subtilis	
RBAM00435	1.7	Similar to unknown proteins from other organisms	AbrB
yheA	1.7	Similar to unknown proteins from B. subtilis	SigD
RBAM03094	1.7	Similar to unknown proteins from other organisms	
yukE	1.7	Similar to unknown proteins from B. subtilis	AbrB, DegU, SigD
ykyA	1.7	Similar to unknown proteins from B. subtilis	
yqzC	1.7	Similar to unknown proteins from B. subtilis	AbrB
yflN	1.7	Similar to unknown proteins from B. subtilis	
yodA	1.8	Similar to unknown proteins from B. subtilis	
ylqC	1.8	Similar to unknown proteins from B. subtilis	AbrB, SigD
engC	1.8	Similar to unknown proteins from B. subtilis	AbrB
RBAM03561	1.8	No similarity	DegU, SigD
yqkC	1.8	Similar to unknown proteins from B. subtilis	AbrB
yrdA	1.8	Similar to unknown proteins from B. subtilis	AbrB
yrkF	1.9	Similar to unknown proteins from B. subtilis	
yaaA	1.9	Similar to unknown proteins from B. subtilis	
yxjC	1.9	Similar to unknown proteins from B. subtilis	SigD
RBAM03268	1.9	Similar to unknown proteins from other organisms	AbrB
yngL	2	Similar to unknown proteins from B. subtilis	AbrB, DegU, SigD, SigM
ypiB	2	Similar to unknown proteins from B. subtilis	AbrB
ymcB	2.1	Similar to unknown proteins from B. subtilis	AbrB
yubD	2.1	Similar to unknown proteins from B. subtilis	
yllB	2.1	Similar to unknown proteins from B. subtilis	DegU, SigD, SigM
ydcD	2.2	Similar to unknown proteins from B. subtilis	DegU, SigD
ypmP	2.2	Similar to unknown proteins from B. subtilis	DegU, SigD
RBAM00520	2.3	Similar to unknown proteins from other organisms	
yqeY	2.5	Similar to unknown proteins from B. subtilis	AbrB
RBAM00434	2.5	No similarity	AbrB, DegU
yviA	1.5	Similar to unknown proteins from B. subtilis	

Appendix Figure 1: A branch of genes which were clustered together according to their transcriptions in response to root exudates. The genes *iolA, iolB, iolC, iolE, iolF,* and *iolG,* which are involved in inositol metabolism, were included in this branch.

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Publikationsliste

Ben Fan, XiaoHua Chen, Anto Budiharjo, Wilfrid Bleiss, Joachim Vater, Rainer Borriss, **Efficient colonization of plant roots by the plant growth promoting bacterium Bacillus amyloliquefaciens FZB42, engineered to express green fluorescent protein,** Journal of Biotechnology, submitted.

Rainer Borriss, XiaoHua Chen, Christian Rueckert, Jochen Blom, Anke Becker, Birgit Baumgarth, **Ben Fan,** Rüdiger Pukall, Peter Schumann, Cathrin Spröer, Helmut Junge, Joachim Vater, Alfred Pühler, Hans-Peter Klenk, **Relationship of *Bacillus amyloliquefaciens* clades associated** 1 **with strains DSM 7^T and FZB42: a proposal for *Bacillus amyloliquefaciens* subsp. *amyloliquefaciens* subsp. nov. and *Bacillus amyloliquefaciens* subsp. *plantarum* subsp. nov. based on their discriminating complete genome sequences,** International Journal of Systematic and Evolutionary Microbiology, accepted.

Oral presentation "**Transcriptional Profiling of *Bacillus amyloliquefaciens* FZB42 Responding Root Exudates**" at the "the *4th European Conference on Prokaryotic Genomics*", Göttingen, Germany, 04.10.2009-07.10.2009.

Berlin, 20.09.2010 **Ben Fan**

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